Olprinone and colforsin daropate alleviate septic lung inflammation and apoptosis through CREB-independent activation of the Akt pathway

Hirofumi Oishi,1,2 Ken-ichi Takano,1 Tomita K, Takebe M, Yokoo H, Yamazaki M, Hattori Y. Olprinone and colforsin daropate alleviate septic lung inflammation and apoptosis through CREB-independent activation of the Akt pathway. Am J Physiol Lung Cell Mol Physiol 303: L130–L140, 2012. First published May 18, 2012; doi:10.1152/ajplung.00363.2011.—Olprinone, a specific phosphodiesterase III inhibitor, and colforsin daropate, a direct adenylate cyclase activator, are now being used in critical conditions. We investigated whether their therapeutic use provides protection against septic acute lung injury (ALI) and mortality. Polymicrobial sepsis was induced by cecal ligation and puncture (CLP) in BALB/c mice. Olprinone or colforsin daropate was continuously given through an osmotic pump that was implanted into the peritoneal cavity immediately following CLP. These treatments prevented the ALI development in CLP mice, as indicated by the findings that severe hypoxemia, increased pulmonary vascular permeability, and histological lung damage were strikingly remedied. Furthermore, continued administration of olprinone or colforsin daropate suppressed apoptosis induction in septic lungs and improved the survival of CLP mice. Olprinone and colforsin daropate enhanced Akt phosphorylation in septic lungs. Wortmannin, which inhibits the Akt upstream regulator phosphatidylinositol 3-kinase, abrogated the protective effects of olprinone and colforsin daropate on sepsis-associated lung inflammation and apoptosis. In vivo transfection of cyclic AMP response element binding protein (CREB) decoy oligodeoxynucleotide failed to negate the abilities of these agents to increase Akt phosphorylation and to inhibit IκBα degradation in septic lungs. These results demonstrate for the first time that CREB-independent Akt-mediated signaling is a critical mechanism contributing to the therapeutic effects of olprinone and colforsin daropate on septic ALI. Moreover, our data also suggest that these cyclic AMP-related agents, by blocking both nuclear factor-κB activation and apoptosis induction, may represent an effective therapeutic approach to the treatment of the septic syndrome.

acute lung injury; adenylate cyclase activator; phosphodiesterase III inhibitor; polymicrobial sepsis

THE SEPSIS SYNDROME IS THE leading cause of death in intensive care units, and its incidence continues to rise (26, 41). Septic shock is a serious complication of sepsis that is associated with hypotension despite adequate fluid resuscitation. Furthermore, acute lung injury (ALI) and multiple organ failure are life-threatening sequelae of sepsis. A major hurdle in the clinical management of patients suffering from the sepsis syndrome is the lack of the effective treatment (41). Thus the important goal in critical care medicine is to find significant therapeutic strategies that will impact favorably on patient outcome.

Cyclic adenosine 3′,5′-monophosphate (cAMP) is an important intracellular signaling molecule that regulates a broad range of cellular processes. Adenylate cyclase catalyzes synthesis of cAMP from ATP, while phosphodiesterases (PDEs) degrade cAMP through its hydrolysis, which would terminate signaling initiated by cAMP formation. The β-adrenergic response is usually blunted during sepsis, and the underlying mechanisms include downregulation of β-adrenoceptors (42). On the other hand, a number of agents that can inhibit PDEs are known to have anti-inflammatory properties (6, 13). It has been shown that these are likely due to their effect on PDE type IV (1, 45, 46, 48). In addition, several lines of evidence have accumulated that PDE type IV inhibitors may be useful in the treatment of ALI (11, 19, 34). However, other members of the PDE superfamily are also abundantly expressed in lung tissues (8, 39), and PDE type III and type V as well as type IV specifically predominate in the lung (40). Moreover, the molecular and cellular mechanisms underlying the anti-inflammatory effects of cAMP-elevating agents are not fully understood.

Olprinone, a specific PDE type III inhibitor, and colforsin daropate, a water-soluble derivative of forskolin that directly stimulates adenylate cyclase, are positive inotropic agents and vasodilators that act beyond β-adrenoceptors to increase intracellular cAMP (21, 36). Both are now being used in critical conditions such as shock or severe circulatory failure. Then, these agents would be of benefit in the treatment of critically ill patients suffering from sepsis if they have the potential to improve the symptoms and outcomes of sepsis. Olprinone has been found to inhibit the sepsis-induced elevation of diaphragmatic cytokine-induced neutrophil chemoattractant-1 (35) and reduce lipopolysaccharide (LPS)-induced pulmonary inflammation (23) in rats. Colforsin daropate has been also shown to attenuate proinflammatory cytokine production and respiratory dysfunction in patients undergoing coronary artery bypass grafting, suggesting its significant anti-inflammatory properties (15).

The aim of the present study was to examine the benefit of olprinone and colforsin daropate in septic ALI and mortality. We used cecal ligation and puncture (CLP) to cause polymicrobial sepsis in mice. CLP-induced sepsis is an animal model that has high clinical relevance to humans, because it reproduces many hallmarks of sepsis that occur in patients (20). We found that olprinone and colforsin daropate mitigated septic lung inflammation and apoptosis and improved the survival of CLP mice. Further studies were then undertaken to gain insight...
into possible mechanism(s) involved in their beneficial effects on septic ALI.

MATERIALS AND METHODS

Model of sepsis. All animal studies were approved by the Animal Care and Use Committee of the University of Toyama. Male BALB/c mice, 8 to 12 wk of age, were quarantined in quiet, humidified, light-cycled rooms for at least 1 wk before use. The surgical procedure to generate CLP-induced sepsis was performed as previously described (32, 47). Mice were anesthetized with 3–4% sevoflurane, and a middle abdominal incision was made. The cecum was mobilized, ligated, and punctured twice with a 21-gauge needle, allowing exposure of feces. The bowel was repositioned, and the abdomen was closed with sterile suture. Sham-operated control mice underwent the same procedure except for ligation and puncture of the cecum. We used a noninvasive computerized tail-cuff system for measuring blood pressure in mice (47).

Drug administration. Microosmotic pumps (Alzet, model 1003D; Durect, Cupertino, CA) delivering olprinone (2 g·kg⁻¹·min⁻¹) or colforsin daropate (1 g·kg⁻¹·min⁻¹) were placed into the peritoneal cavity before the abdomen was closed. The placebo group was administered an equal volume of saline. When wortmannin (BIOMOL International, Plymouth Meeting, PA) was used, the animals were treated with 1 mg/kg of wortmannin intravenously twice a day from 1 h after CLP.

Preparation and transfection of decoy ODNs. The CREB (cAMP response element binding protein) decoy oligodeoxynucleotide (ODN) sequences are 5'-TGC TAT GTG ACA ATG G-3' and 5'-CCA TTC TTT TG TAC AGC A-3', whereas the scrambled decoy ODN sequences are 5'-TGC TAT GCC CCT TTT AGA ATG G-3' and 5'-CCA TTC TAA AAG GGG CAT AGC A-3'. The hemagglutinating virus of the Japan envelope vector system (HVJ Envelope Vector Kit GenomONE-Neo; Ishihara Sangyo, Osaka, Japan) was used for in vivo gene transfer (31). Sterile saline containing the HVJ-liposome complex (120 μg of encapsulated ODN) was infused into the tail vein over 5 s at room temperature 60 min after sepsis induction.

Enzyme immunoassay for cytokines. Blood levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were measured by the use of commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The plate was read on a microplate reader (Nippon-InterMed, Tokyo, Japan). Assays were performed in duplicate.

Evaluation of bacterial clearance. Bacterial loads were determined in peritoneal lavage. In brief, 24 h after CLP procedures, 1 ml of sterile normal saline was injected into the peritoneal space and mixed
thoroughly by gentle massage of the abdomen. Then, 100 µl of the peritoneal lavage were collected. Samples were serial diluted, placed on MacConkey agar medium (Eiken Chemical, Tokyo, Japan), and incubated at 37°C for 24 h. Colony-forming units were counted and expressed as log10 of colony-forming unit per milliliter of lavage fluid.

**Pulmonary microvascular leakage.** Lung vascular leak was assessed by Evans blue dye extravasation as described previously (47). In brief, Evans blue dye (20 mg/kg) was given intravenously to mice 30 min before the animals were killed. The lungs were perfused with heparinized saline and two samples of lung parenchyma were removed. Both were weighed, and then one was placed in formamide and the other was put in an oven (60°C) to dry overnight. The absorbance of Evans blue dye extracted in formamide was measured by spectrophotometry at a wavelength of 620 nm. The concentration of the Evans blue dye then was calculated from a standard curve and expressed as micrograms of Evans blue dye per gram dry weight of tissue.

**RNA extraction and quantitative real-time PCR.** Total RNA was isolated from lung tissue with the use of RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. RNA was reverse-transcribed to cDNA, and real-time PCR was performed in a final volume of 20 µl containing cDNA template and primers by using a Takara RNA PCR kit (Takara Shuzo, Ohtsu, Japan) as described in the manufacturer’s manual. The sequences of specific primer pairs for target genes are available upon request from a website of Takara Bio (http://www.takara-bio.co.jp/). The PCR program consisted of 95°C for 30 s for initial denaturation of DNA, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s for annealing of primers, and 95°C for 1 min for elongation. GAPDH served as an internal control for normalization. Relative expression was calculated according to the $2^{-ΔΔCt}$ method as previously described (25).

**Electrophoretic mobility shift assay.** Nuclear protein extracts from freshly isolated lungs were obtained with a commercially available nuclear extraction kit (Sigma-Aldrich, St. Louis, MO) as described in the manufacturer’s manual. Electrophoretic mobility shift assays were carried out with Odyssey Infrared electrophoretic mobility shift assay kit (LI-COR, Lincoln, NE) according to the manufacturer’s instructions. Double-stranded IRDye 700 infrared dye-labeled oligonucleotides with consensus sequences of nuclear factor-κB (NF-κB) (5’-AGT TGA GGG GAC TTT CCC AGG C-3’ and 3’-TCA ACT CCC G-5’), and CREB (5’-AGA GAT TGC CTG AGC TCA GAG AGC TAG-3’ and 3’-TCT CTA GAC TGC AGT CTC TCG ATC-5’) were used.

**Western blot analysis.** Samples of homogenate (60 µg of protein) were run on 12% or 15% SDS polyacrylamide gels. Blotting procedure, chemiluminescent detection, and densitometric analysis were performed as previously described by our laboratory (22, 29). Membranes were probed with anti-IκBα (Cell Signaling, Danvers, MA), anti-cleaved caspase-3 (Cell Signaling), anti-Akt (Cell Signaling), anti-phospho-Akt (Ser-473) (Cell Signaling), or anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

**Histological examination.** For routine histology, inflation-fixed lungs were harvested, fixed, dehydrated, paraffin-embedded, and sliced into 4-µm-thick sections (30). After deparaffinization, slides were stained with hematoxylin and eosin by standard methods. A semiquantitative morphometric analysis of lung injury was performed by scoring from 0 to 4 (none, light, moderate, severe, very severe) for the following categories: neutrophil infiltration, pulmonary edema, and disorganization of lung parenchyma and hemorrhage. A total lung injury score was calculated by adding the individual scores in every animal and averaging the total values in each group. All the histological studies were performed in a blinded fashion.

**Assessment of apoptosis.** Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) was performed using the ApoTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Additional details are described elsewhere (22).

**Statistical analysis.** The data are presented as means ± SE ($n = 3–15$). Data were analyzed by use of Prism software (ver. 4; GraphPad Software, San Diego, CA). Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison test. A $P$ value less than 0.05 was considered significant.

**RESULTS**

**Effects on hypotension, mortality, blood cytokine levels, and bacterial clearance after CLP.** When polymicrobial sepsis was induced by CLP in mice, a progressive fall in blood pressure was observed (Fig. 1A). At 24 h after surgery, mean arterial blood pressure was significantly lower in the CLP group than in the sham group (43 ± 8 vs. $80 ± 5$ mmHg, $n = 3$, $P < 0.001$). Some animals were continuously given olprinone (2 µg·kg$^{-1}$·min$^{-1}$) or colforsin daropate (1 µg·kg$^{-1}$·min$^{-1}$) through an osmotic pump that was implanted into the peritoneal cavity immediately following CLP. Neither olprinone nor colforsin daropate affected the CLP-induced hypotensive response despite the fact that they have a vasodilating property.

CLP mice showed a sharp decline in survival in the course of 2 days. Treatment of CLP mice with olprinone or colforsin daropate significantly improved survival compared with CLP alone ($P < 0.01$). Both olprinone and colforsin daropate significantly improved survival compared with CLP alone (Fig. 2A).

**Assessment of lung vascular permeability.** Lung vascular permeability was assessed by Evans blue dye extravasation at 24 h after sham operation or CLP. Evans blue dye extravasation was quantified by measurement of arterial PO$_2$, and blood gas analysis was performed in mice 24 h after sham operation or CLP. The blood samples were taken from the abdominal aorta ($n = 4–8$ for each group). **$P < 0.01$ compared with sham-operated control. ***$P < 0.001$ compared with CLP alone. B: lung vascular permeability was assessed by Evans blue dye extravasation at 24 h after sham operation or CLP in mice given saline, olprinone, or colforsin daropate ($n = 3–4$ for each group). ***$P < 0.001$ compared with sham-operated control. ###$P < 0.001$ compared with CLP alone.**
daropate during the first 3 days resulted in significantly improved survival \( (P < 0.05, \) log rank test) (Fig. 1B).

When blood levels of proinflammatory cytokines were measured via an enzyme-linked immunosorbent assay, the sham-operated control animals had very low levels of the cytokines examined here. After induction of sepsis by CLP, the proinflammatory cytokines TNF-\( \alpha \), IL-1\( \beta \), and IL-6 showed a marked increase. Treatment with olprinone or colforsin daropate significantly inhibited their increased levels at 12 h after CLP-induced sepsis (Fig. 1C).

We further examined the bacterial loading in the peritoneal lavage from mice 16 h after sham or CLP surgery. No bacteria grew in any of the sham samples (data not shown). All of the mice subjected to CLP surgery exhibited substantial bacterial counts in the peritoneal lavage (Fig. 1D). Notably, there was no difference among the three groups of mice regardless of whether olprinone or colforsin daropate was administered, indicating that olprinone and colforsin daropate have a minimal effect on the host bacterial clearance during polymicrobial sepsis.

**Effects on sepsis-associated lung inflammation.** When blood gases were measured in arterial blood samples from mice 24 h after the onset of CLP-induced sepsis, septic mice displayed a severe hypoxemic condition, as indicated by markedly reduced \( \text{Po}_2 \), compared with sham control mice (Fig. 2A). This hypoxemia associated with sepsis was profoundly improved by continued administration of olprinone or colforsin daropate.

CLP-induced sepsis showed an approximately threefold increase in lung microvascular permeability, as assessed by Evans blue dye extravasation (Fig. 2B). Treatment with olprinone or colforsin daropate resulted in a reduction in lung microvascular permeability to the level observed in the sham-operated control group.

Light microscopy findings revealed that massive infiltration of inflammatory cells and thickening of the alveolar septum were observed in lungs from CLP-induced septic mice compared with sham-operated control lungs (Fig. 3A). In lungs from septic animals that were given olprinone or colforsin daropate, architecture of alveoli was preserved with less infiltration of inflammatory cells. Semiquantitative assessment using lung injury score showed that the score was significantly lowered when CLP mice were treated with olprinone or colforsin daropate.

We also examined changes in the levels of mRNA of inflammatory mediators in septic lungs using real-time PCR (Fig. 4). After induction of sepsis by CLP, mRNA expression

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**Fig. 3.** Lung sections stained with hematoxylin and eosin. Mice underwent sham procedure, CLP, CLP and olprinone administration, or CLP and colforsin daropate administration. Tissues were harvested 24 h after surgery. A: representative micrographs. Magnified view is shown in each bottom panel. B: semiquantitative analysis of lung tissues by lung injury score. ***\( P < 0.001 \) compared with sham-operated control. ####\( P < 0.001 \) compared with CLP alone.
levels of the proinflammatory cytokines, TNF-α, IL-1β, and IL-6, were increased about three-, four-, and ninefold, respectively. Sepsis also resulted in a huge increase (60-fold) in gene expression of MCP-1 (monocyte chemoattractant protein-1), a chemokine that triggers the infiltration and activation of cells of the monocyte-macrophage lineage. Furthermore, the transcript levels of iNOS (inducible nitric oxide synthase) and ICAM-1 (intercellular adhesion molecule 1) were about nine- and sixfold higher in septic than in control lungs, respectively. The sepsis-induced increases in mRNA expression of these inflammatory mediators were significantly suppressed by olprinone or colforsin daropate.

Effects on apoptosis in septic lungs. The tissue sections were labeled with an in situ TUNEL assay to detect apoptotic cells in lungs (Fig. 5A). No TUNEL-positive cells were detectable in sham-operated control mice. Induction of sepsis by CLP resulted in a marked appearance of TUNEL-positive cells. Apoptotic cells were identical morphologically to endothelial cells of capillary vessels in the alveolar septa and to epithelial type II cells (32). In lungs from CLP mice given olprinone or colforsin daropate, TUNEL-positive cells were strikingly decreased. These results of quantitative analysis are summarized in Fig. 8C.

Furthermore, Western blot analysis of the cleaved form of caspase-3, a member of the caspase superfamily that initiates cell apoptotic events, revealed that the increase in its pulmonary expression level in CLP mice was prevented by treatment with olprinone or colforsin daropate (Fig. 5B).

Effects on NF-κB activity in sepsis. To examine the activation of NF-κB in septic lungs, analysis of NF-κB binding activity was performed in nuclear protein extracts from lungs by gel mobility shift assays (Fig. 6A). The DNA binding activity of NF-κB was greatly increased in lung nuclear extracts from mice at 12 h after CLP. This increase was strongly eliminated by continued administration of olprinone or colforsin daropate. Furthermore, since the activity of NF-κB is primarily regulated by interaction with its inhibitory protein IκB, degradation of IκB was monitored by Western blot analysis (Fig. 6B). Treatment with olprinone or colforsin daropate significantly inhibited IκB degradation seen in septic lungs.

Akt activation and counteraction by wortmannin. Activation of the serine/threonine kinase Akt was assessed by Western blot analysis for phospho-Akt at Ser473 (Fig. 7A). Akt phosphorylation transiently increased in lungs at 3 h after CLP. However, its phosphorylation levels returned to the baseline value and were not different from control between 6 and 24 h after CLP. Total Akt levels were unaffected by sepsis; this result served to confirm equal protein loading on Western blot analysis. Treatment with olprinone or colforsin daropate led to significant increases in Akt phosphorylation in lungs at both 3 and 12 h after CLP (Fig. 7, B and C). Wortmannin is a widely used inhibitor of phosphatidylinositol 3-kinase (PI3K). Because Akt is located downstream of PI3K, Akt functions as part of a wortmannin-sensitive pathway. Wortmannin treatment had no effect on lung histology.
and the presence or absence of apoptosis in sham-operated control animals (Fig. 8, A and B). However, when wortmannin was given to CLP mice, this treatment resulted in an abrogation of the olprinone- or colforsin daropate-induced improvement of the histological damage in the lung (Fig. 8A). In agreement with canceling the anti-inflammatory effects of olprinone and colforsin daropate, their preventive effects on IκBα degradation in septic lungs was reversed by wortmannin (Fig. 8D). Moreover, wortmannin treatment negated their protective effects on pulmonary cell apoptosis mediated by sepsis (Fig. 8, B and C).

No counteraction by CREB decoy ODN. As shown in Fig. 9A, both olprinone and colforsin daropate led to enhanced CREB phosphorylation at Ser133 in CLP-induced septic lung tissues. Notably, the phosphorylated level of CREB was greatly declined in untreated sepsis. Furthermore, the binding activity of CREB, as assessed by gel mobility shift assay, was appreciable in septic lungs and was evidently increased when CLP mice were treated with olprinone or colforsin daropate (Fig. 9B).

Transfection of CREB decoy ODN, but not of scrambled decoy ODN, via intravenous injection at 1 h after CLP, resulted in a disappearance of the binding of CREB in lung tissues from the animals given olprinone or colforsin daropate (Fig. 9B). This suggests our successful in vivo transfer of CREB decoy ODN to efficiently eliminate the activity of this transcriptional factor in lung tissues of CLP mice. However, transfection of CREB decoy ODN did not substantially alter the increasing effects of olprinone and colforsin daropate on Akt phosphorylation in lungs of CLP mice (Fig. 9C). In addition, CREB decoy ODN treatment was without effect on the inhibition by the two agents of IκBα degradation in septic lungs (Fig. 9D).

DISCUSSION

In the present study, we demonstrated that continued administration of olprinone, a phosphodiesterase III inhibitor, or colforsin daropate, an adenylate cyclase activator, resulted in a significant improvement of ALI in mice with CLP-induced sepsis. In CLP-induced septic mice, marked hypoxemia, increased lung vascular permeability, and severe histological damage in lungs were observed. These pathophysiological consequences of ALI were greatly prevented when olprinone or colforsin daropate therapy was initiated immediately follow-

Fig. 5. Effects of treatment with olprinone and colforsin daropate on sepsis-associated cell apoptosis in lungs. Mice underwent sham procedure, CLP, CLP and olprinone administration, or CLP and colforsin daropate administration. A: lung tissue sections by an in situ terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay. Tissues were harvested 24 h after surgery. Red arrowheads indicate TUNEL-positive apoptotic cells. B: Western blot analysis was performed using anti-cleaved caspase-3 antibodies. Lung samples were collected 12 h after surgery. No apparent difference in GAPDH, which served as loading control, among groups was noted. The summarized data were expressed as a fold increase above control (sham operation) normalized to GAPDH (n = 5–6 for each group). **P < 0.01, ***P < 0.001 compared with sham-operated control. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with CLP alone.
Apoptosis is an important prominent feature of sepsis (14). Lymphocytes, parenchymal cells (including intestinal and lung epithelial cells), and vascular endothelial cells have increased apoptotic cell death in animal models of septic shock (2, 12, 17). To assess whether treatment with olprinone or colforsin daropate affects sepsis-induced apoptotic cell death in lungs, the tissue sections were labeled with an in situ TUNEL assay. Induction of sepsis by CLP resulted in a marked appearance of TUNEL-positive cells in lung sections, but TUNEL-positive cells were sharply reduced when CLP mice were continually given olprinone or colforsin daropate. Additionally, the sepsis-associated increase in cleaved caspase-3 in lungs was prevented by treatment with olprinone or colforsin daropate. These findings provide protective effects of olprinone and colforsin daropate on sepsis-induced cell apoptosis in lungs. Recent accumulating evidence suggests that cell apoptosis may be potentially detrimental in septic ALI (5). Interestingly, studies using transgenic and gene knockout mice, providing protection against apoptotic cell death, have demonstrated that these animals can display a significant survival benefit in CLP-induced sepsis (4, 18). Furthermore, we and other laboratories have recently reported that gene silencing with small interfering RNA therapy, aimed at blocking cell signaling pathways leading to apoptosis, dramatically improves the survival of septic mice (32, 51). It is thus most likely that the antiapoptotic actions of olprinone and colforsin daropate are one of the principal mechanisms by which these agents improved septic mortality.

We found that CLP-induced sepsis resulted in a transient increase in Akt phosphorylation in lungs followed by a return to base line within 6 h. According to in vitro studies, LPS stimulation leads to phosphorylation of Akt in human alveolar macrophages (37), human monocytic cells (10), and human umbilical vein endothelial cells (7), although the time dependence of LPS-induced Akt phosphorylation for each is quite different. Meanwhile, Veres et al. (50) have...
reported a moderate increase in Akt phosphorylation in the heart, liver, and small intestine of LPS-treated mice. Thus the degree of reactive phosphorylation of Akt may vary somewhat depending on cell/tissue types and endotoxic conditions being studied. Here we demonstrated that continued administration of olprinone or colforsin daropate caused a significant increase in Akt phosphorylation in lungs of CLP-induced septic mice. Akt is a member of the PI3K signal transduction enzyme family, which can regulate cellular activation, inflammation, chemotaxis, and apoptosis (3). When phosphorylated by its upstream regulator PI3K, Akt prevents apoptotic cell death in a variety of settings (3). In this study, wortmannin, a specific inhibitor of PI3K, abrogated the abilities of olprinone or colforsin daropate to reduce lung inflammation and apoptosis in septic mice. Wortmannin also abrogated the inhibition by olprinone or colforsin daropate of sepsis-induced IkBα degradation, suggesting that their protective effects on IkBα may be a result of the activation of the PI3K/Akt pathway. We thus propose that olprinone and colforsin daropate lead to phosphorylation and hence activation of Akt. Furthermore, we believe that, downstream of Akt, many of the beneficial effects of these agents observed in septic mice may be caused by the inhibition of the central proinflammatory transcription factor NF-κB and of the apoptotic machinery.

Both olprinone and colforsin daropate are agents that raise intracellular levels of cAMP, which in turn activates cAMP-dependent protein kinase A (PKA). PKA phosphorylates and activates CREB, a transcription factor that regulates positively or negatively expression of numerous genes (44). CREB activity can be stopped by dephosphorylation. Although there is one previous report which showed that LPS injection upregulated the DNA binding activity of CREB in rat lungs (52), we observed that sepsis induction by CLP resulted in reduced phosphorylation and decreased DNA binding activity of CREB in mouse lungs. Treatment with olprinone or colforsin daropate caused resumption of phosphorylation of CREB and thereby increased CREB DNA-binding activity in septic lungs. Treatment with olprinone or colforsin daropate caused resumption of phosphorylation of CREB and thereby increased CREB DNA-binding activity in septic lungs. However, the cAMP/PKA/CREB pathway is unlikely to be responsible for Akt activation by olprinone and colforsin daropate. We found that systemic delivery of CREB decoy ODN, which successfully eliminated CREB DNA-binding activity increased by these agents in septic lungs, was without effect on their increases in Akt phosphorylation. Furthermore, the inhibitory effects of the two agents on sepsis-associated degradation of IkBα was unaffected by CREB decoy ODN transcription. In addition to acting through PKA, cAMP also activates Epac proteins. The novel cAMP mediators Epac1 and Epac2 are now assumed to control a range of diverse effectors and to regulate a number of pivotal processes, including cell survival and apoptosis (9). A recent report has
demonstrated that activation of Epac leads to an increase in Akt phosphorylation in neuronal cells, whereas PKA activation has the opposite effect (38). In contrast, another report indicates that a synergistic action of Epac and PKA is responsible for cAMP inhibition of Akt in thyroid cells (16). To date, thus, the exact role of Epac in the connection of cAMP-dependent signaling with downstream Akt activation still remains unsettled.

We showed that treatment with olprinone or colforsin daropate significantly inhibited the elevations of circulating levels of pro-inflammatory cytokines during sepsis, suggesting that these treatments were able to calm the systemic inflammatory response. We also found that olprinone and colforsin daropate prevented apoptosis of not only pulmonary cells but spleen lymphocytes in sepsis (unpublished observations). These salutary effects may contribute to a reduction in septic mortality by ameliorating the multiple organ dysfunction. However, the present experiments do not allow further speculation as to the precise mechanism(s) underlying the survival benefit of olprinone and colforsin daropate in mice with CLP-induced sepsis. Furthermore, it is not clear at this time what cells receive a benefit from olprinone and colforsin daropate in this murine model of sepsis. In addition, one may argue that administration of olprinone or colforsin daropate through an osmotic pump that was implanted into the peritoneal cavity immediately following CLP does not accurately represent the clinical situation. The investigation of treatment after sepsis induction would have the additional clinical value. These limitations of the present study are the subject of ongoing research in this laboratory.

In conclusion, we show the first evidence that olprinone and colforsin daropate may confer novel therapeutic potential in septic ALI. Our study highlights that the protective effects of these cAMP-related agents on lung inflammation and apoptosis in sepsis are attributable to CREB-independent activation of the Akt pathway. While appreciating that additional work is required to optimize clinical application, we suggest that the present study could provide a novel mechanistic insight into their usefulness as a therapeutic tool for the treatment of septic ALI.

Fig. 8. Wortmannin counteracts the beneficial effects of olprinone and colforsin daropate in sepsis-associated derangements in lungs. Whereas olprinone or colforsin daropate was continuously administered immediately after CLP, wortmannin was intravenously injected twice a day from 1 h after CLP. A: tissue sections stained with hematoxylin and eosin. Sections were harvested 24 h after surgery. B: micrographs showing apoptotic cells by an in situ TUNEL assay. Red arrowheads indicate TUNEL-positive apoptotic cells. Sections were prepared from mice 24 h after surgery. C: estimation of apoptotic cells by an in situ TUNEL assay (n = 3–6). Counts of apoptotic cells were made in the sections at a final magnification of ×200, and the average of apoptotic cell number in 3 middle-power fields per samples was calculated. *P < 0.05, **P < 0.01, ***P < 0.001. D: Western blots showing that wortmannin reduced the increase in IκBα protein by olprinone or colforsin daropate in lung tissues 24 h after surgery. GAPDH served as loading control. This experiment was repeated twice.
Fig. 9. cAMP response element binding protein (CREB) decoy oligodeoxynucleotide (ODN) does not counteract the effects of olprinone and corforsin daropate on Akt phosphorylation and IκBα degradation in septic lungs. A: Western blot showing a decrease in the phospho-CREB level at 6 h after sepsis induction. Total CREB was used as a loading control. B: gel mobility shift assay for CREB binding activity in lung tissues. The induced CREB shift bands are indicated. Lane 1, free probe showed no detection of CREB binding activity; lane 2, nuclear extracts from control lung tissues 12 h after sham operation are incubated with an IRDye 700 infrared dye-labeled CREB probe; lane 3, nuclear extracts were taken from tissues 12 h after CLP; lane 4, nuclear extracts were taken from tissues of CLP mice given olprinone; lane 5, nuclear extracts were taken from tissues of CLP mice given corforsin daropate; lane 6, nuclear extracts were taken from tissues of olprinone-treated mice that were transfected with CREB decoy ODN 60 min after CLP; lane 7, nuclear extracts were taken from tissues of corforsin daropate-treated mice that were transfected with CREB decoy ODN 60 min after CLP; lane 8, nuclear extracts were taken from tissues of olprinone-treated mice that were transfected with scrambled decoy ODN 60 min after CLP; lane 9, nuclear extracts from CLP mice tissues were incubated with a dye-labeled CREB probe in the presence of excess unlabeled CREB ODNs. C: phospho-Akt levels by Western blotting. The summary of quantification of densitometric measurements is presented as ratio of phospho-Akt relative to total Akt (n = 4 – 6). D: IκBα protein levels by Western blotting. The summary of quantification of densitometric measurements is presented as ratio of IκBα relative to GAPDH (n = 4 – 6). ***P < 0.001 compared with sham-operated control. #P < 0.05, ##P < 0.01 compared with CLP alone. In C and D, lung samples for Western blotting were collected 12 h after surgery.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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