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Akt pathway is required for oestrogen-mediated attenuation of lung injury in a rodent model of cerulein-induced acute pancreatitis

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ABSTRACT

Background: The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) is known to be an endogenous negative feedback or compensatory mechanism that serves to limit pro-inflammatory and chemotactic events in response to injury. The aim of this study is to elucidate whether Akt plays any role in 17β-estradiol (E2)-mediated attenuation of lung injury after acute pancreatitis (AP).

Materials and methods: Male Sprague–Dawley rats underwent cerulein-induced AP. Rats were treated with vehicle (cyclodextrin), E2 (1 mg/kg body weight [BW]), or E2 plus PI3K/Akt inhibitor Wortmannin (100 μg/kg BW) 1 h after the onset of AP. At 8 h after sham operation or AP, various parameters were measured.

Results: AP led to a significant decrease in lung Akt phosphorylation, which was associated with increased lung tissue myeloperoxidase (MPO) activity, wet-to-dry weight ratios, interleukin (IL)-6, tumor necrosis factor (TNF)-α, cytokine-induced neutrophil chemoattractant (CINC)-1, and CINC-3 levels. Administration of E2 after AP restored the AP-induced decrease in Akt phosphorylation and attenuated the increase in lung injury markers (MPO activity and wet-to dry weight ratios) and pro-inflammatory mediator production. The effects of E2 on the lung were abolished by co-administration of Wortmannin.

Conclusions: These results collectively suggest evidences that the Akt pathway seems to be required for E2-mediated protection of lung injury after AP.

Introduction

Acute pancreatitis (AP) is a non-infectious inflammatory reaction of the pancreas, followed by systemic organ damage.[5] Lung injury except for pancreatic damage, renal dysfunction or cardiovascular compromise is a frequent presentation after AP.[5,15] Acute lung injury and acute respiratory distress syndrome account for about 60% of all deaths in the early stage of severe AP.[2] Furthermore, multiple organ failure or dysfunction secondary to a systemic inflammatory response remains the major cause of mortality and morbidity following AP.[3,16,18] The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) is known to be an endogenous negative feedback or compensatory mechanism that serves to limit pro-inflammatory and chemotactic events in response to injury.[17,44] Inhibition of PI3K/Akt pathway with a PI3K inhibitor increases serum cytokines levels and decreases the survival of mice subjected to sepsis.[28,44] Furthermore, studies have shown that PI3K/Akt plays a protective role in myocardium or cardiomyocytes responses to injury.[28,43,47]

It is well-established that gender can influence immune and organ functions after injury.[10,11,22,45] Furthermore, findings from clinical and experimental conditions suggest that females are more tolerant to injury than males.[12,33,45] In this regard, previous studies have shown that administration of 17-β estradiol (E2) following trauma–haemorrhage or hypoxia prevents lung damage in male rats.[27,32,34] PI3K/Akt signalling cascade by E2 has been observed in different cells/tissues.[21,24,48] For example, E2 induced the activation of Akt in rat cardiomyocytes and vascular endothelial cells.[21,47] Other investigators have indicated that the neuroprotective effects of E2 on glutamate-induced neuron toxicity in rat neurons are through the phosphorylation of Akt.[21] Furthermore, it has been reported that human endothelial cells stimulated with E2 increases PI3K activity, leading to the activation of Akt.[39] Nonetheless, it remains unknown whether administration of E2

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following AP has any salutary effects on the lung and whether Akt is associated with E2-mediated attenuation of lung injury under those conditions. To test these hypotheses, we treated male animals with E2 or E2 plus Akt inhibitor Wortmannin following cerulein-induced AP. The effects of E2 were then examined on lung tissue wet-to-dry weight ratios, myeloperoxidase (MPO) activity, phospho-Akt/Akt, interleukin (IL)-6, tumor necrosis factor (TNF)-α, cytokine-induced neutrophil chemoattractant (CINC)-1, and CINC-3 levels following cerulein-induced AP.

Materials and methods

Rat cerulein-induced acute pancreatitis (AP) model

Male (325–375 g) Sprague–Dawley rats, were maintained in an animal room with 12 h:12 h light–dark cycle (lights on from 8:00 AM to 8:00 PM) and an ambient temperature of 22 ± 1 °C. Animals were fasted for 4 h before the experiment but had free access to water. The use of experimental animals and procedures used in this study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. A rat model of cerulein-induced AP used here was the same as that described previously.[7,8] At the time of the experiment, rats were anaesthetised by administration of ketamine chloride (100 mg/kg) intraperitoneally (ip) and the incision wound was bathed with xylocaine. The mean arterial blood pressure (BP) was measured and recorded by a BP analyser (DigiMed, Louisville, KY) connected to a polyethylene catheter (PE-50, Becton Dickinson, Sparks, MD) placed in the femoral artery. The left femoral vein was catheterised for continuous infusion of normal saline at a rate of 4 ml/kg/h. Anaesthesia was maintained with intravenous ketamine chloride at a rate of 40 mg/kg/h. The animals were allowed to breathe room air spontaneously, and their rectal temperature was maintained at 37 °C with a heating pad. To induce AP, cerulein (Sigma, St. Louis, MO) was administered intravenously (iv) to the maintained fluid at a dose of 15 μg/kg/h. In sham-operated rats, vehicle (cyclodextrin, Sigma, iv) was given. In AP animals, vehicle, E2 (1 mg/kg, Sigma, iv) or E2 plus PI3K/AKT inhibitor Wortmannin (100 μg/kg, Sigma, ip) was administered at 1 h following AP. Each experimental group had 4–5 rats. The animals were sacrificed 8 h thereafter, and the lung was harvested and stored at −70 °C freezer.

Measurement of lung wet-to-dry weight ratios

Wet/dry weight ratios of lung tissue were used as a parameter of oedema formation. Tissue samples (right lobe of the lung) were weighted immediately after removal (wet weight) and then subjected to desiccation in an oven at 80 °C for 48 h. The ratios of the wet-dry weight were then calculated.

Measurement of lung myeloperoxidase (MPO) activity

MPO activity in homogenates of left lung tissue was determined as described previously.[23] All reagents were purchased from Sigma Chemical Co. Briefly, equal weights (100 mg wet weight) of lung from various groups were suspended in 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) and sonicated at 30 cycles, twice, for 30 s on ice. Homogenates were cleared by centrifuging at 17,000 × *g* at 4 °C for 10 min, and the supernatants were stored at −70 °C freezer. Protein content in the samples was determined using the Bio-Rad D_C Protein Assay (Bio-Rad Laboratories, Hercules, CA). The samples were incubated with a substrate o-dianisidine hydrochloride. This reaction was carried out in a 96-well plate by adding 290 μl 50 mM phosphate buffer, 3 μl substrate solution (containing 20 mg/ml o-dianisidine hydrochloride), and 3 μl H_2O_2 (20 mM). Sample (10 μl) was added to each well to start the reaction. Standard MPO (Sigma) was used in parallel to determine MPO activity in the sample. The reaction was stopped by adding 3 μl sodium azide (30%). Light absorbance at 460 nm was read. MPO activity was determined by using the curve obtained from the standard MPO.

Western blot analysis

Approximately 0.1 g of freshly collected left lung tissue from each rat was homogenised in 1 ml of lysis buffer containing 50 mM HEPES, 10 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.15 M NaCl, 0.1 M NaF, 10% glycerol, 0.5% Triton X-100 and protease inhibitor cocktail (Sigma). Tissue lysates were centrifuged at 17,000 × *g* for 20 min at 4 °C and an aliquot of the supernatant was used to determine protein concentration (Bio-Rad D_C Protein Assay). The lysates (50 μg per lane) were then mixed with 4× sodium dodecyl sulfate (SDS) sample buffer and were electrophoresed on 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred electrophoretically onto nitrocellulose membranes (Invitrogen). The membranes were immunoblotted with the following primary antibodies against Akt, phospho-Akt (Cell Signaling Technology, Beverley, MA). Rabbit polyclonal β-actin antibody (Abcam, Cambridge, MA) was used to determine β-actin as the loading control by stripping the same targeted membranes. The membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody for detection of bound antibodies by enhanced chemiluminescence (Amersham, Piscataway, NJ).

Determination of lung IL-6, TNF-α, CINC-1, and CINC-3 levels

Levels of IL-6, TNF-α, CINC-1, and CINC-3 levels in the left lung tissue were determined using enzyme-linked immunosorbent assay kits (R&D, Minneapolis, MN) according to the manufacturer's instructions. The same supernatant (100 μl) of the Western blot was used for determination of IL-6, TNF-α, CINC-1, and CINC-3 levels. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad D_C Protein assay). The concentration of these pro-inflammatory mediators is expressed pg/mg protein in each sample.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's test were employed for the comparison among groups ($n = 4–5$), and differences were considered significant at $p < 0.05$.

Results

Alterations of lung tissue oedema

As shown in Fig. 1, there was a marked increase in lung tissue wet/dry weight ratios following AP rats as compared to sham-operated rats, suggesting that AP increases water content in the lung tissue. Administration of E2 prevented the AP-induced increase in lung wet/dry weight ratios, which was abolished by co-administration of PI3K/AKT inhibitor Wortmannin.

Lung tissue MPO activity

Lung tissue MPO activity was increased in vehicle-treated AP rats compared to shams (Fig. 2). The increase in lung tissue MPO activity after AP was normalised by E2. Co-administration of Wortmannin abolished the E2-mediated decrease in MPO activity following AP.

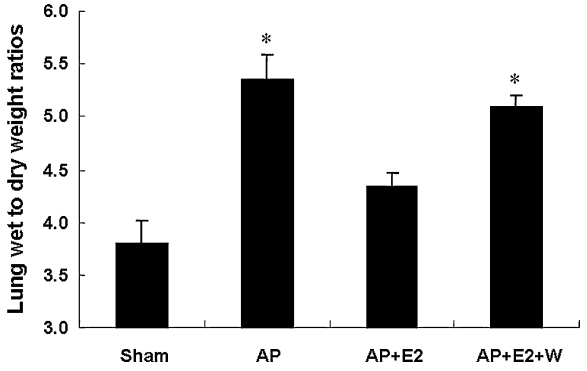

Fig. 1. Lung tissue wet/dry weight ratios at 8 h after sham or acute pancreatitis (AP). Animals were treated with vehicle, 17β-estradiol (E2), or E2 plus Wortmannin (W). Data are shown as mean ± SEM of 4–5 animals in each group. $^{*}p < 0.05$ vs. sham or AP + E2.

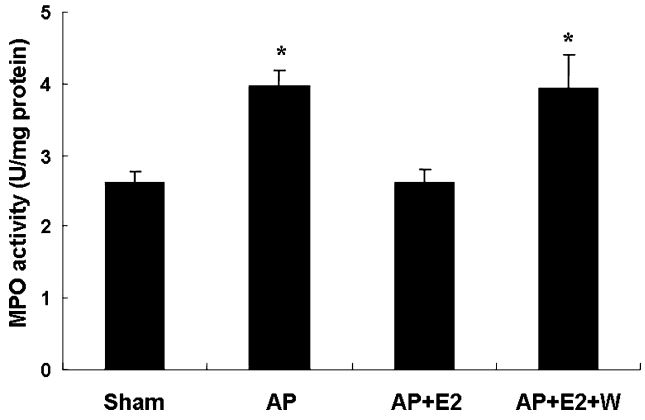

Fig. 2. Lung tissue myeloperoxidase (MPO) activity at 8 h after sham or acute pancreatitis (AP). Animals were treated with vehicle, 17β-estradiol (E2), or E2 plus Wortmannin (W). Data are shown as mean ± SEM of 4–5 animals in each group. $^{*}p < 0.05$ vs. sham or AP + E2.

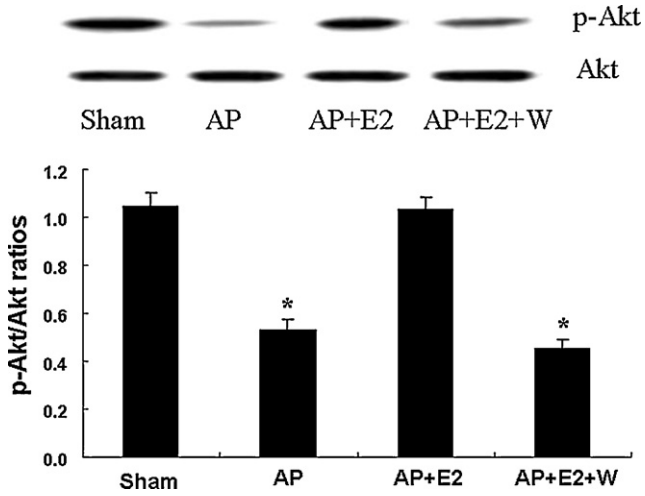

Fig. 3. Expression of total and phosphorylated (activated) protein kinase B (p-Akt) in the lung at 8 h after sham or acute pancreatitis (AP). Animals were treated with vehicle, 17β-estradiol (E2), or E2 plus Wortmannin (W). Blots obtained from several experiments were analysed using densitometry. The densitometric values were pooled from animals in each group and are shown as mean ± SEM of 4–5 animals in each group. $^{*}p < 0.05$ vs. sham or AP + E2.

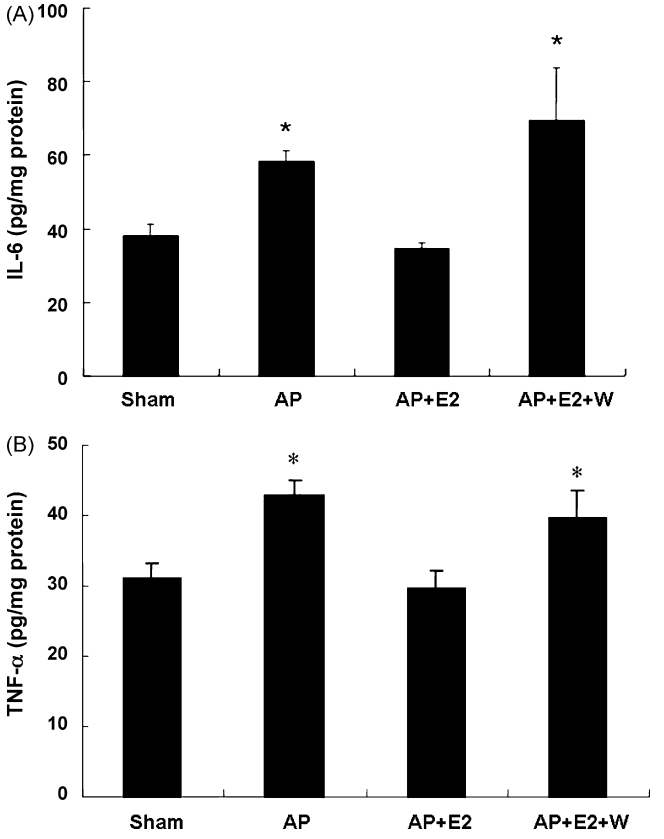

Fig. 4. Levels of lung interleukin (IL)-6 (A) and (TNF)-α (B) at 8 h after sham or acute pancreatitis (AP). Animals were treated with vehicle, 17β-estradiol (E2), or E2 plus Wortmannin (W). Data are shown as mean ± SEM of 4–5 animals in each group. $^{*}p < 0.05$ vs. sham or AP + E2.

Activation of lung Akt

AP resulted in a significant decrease in Akt phosphorylation as compared to shams (Fig. 3). Administration of E2 restored the AP-induced reduction in Akt phosphorylation, which was blocked by co-administration of Wortmannin (Fig. 3). There was no change in total lung Akt protein expression among sham-operated and AP rats.

Lung IL-6 and TNF-α contents

Levels of IL-6 and TNF-α in the lung tissue (Fig. 4A and B) were significantly elevated following AP compared to shams. Administration of E2 normalised the AP-induced increase in levels of IL-6 and TNF-α, which were abolished by co-administration of Wortmannin.

Lung CINC-1 and CINC-3 concentrations

Levels of lung CINC-1 and CINC-3 (Fig. 5A and B) were increased in AP rats treated with vehicle compared to shams. Administration of E2 after AP decreased lung CINC-1 and CINC-3 levels to values that were even higher than shams. However, the decrease in lung CINC-1 and CINC-3 levels by E2 after AP was abolished by co-administration of Wortmannin.

Discussion

This study was performed to determine the role of Akt in E2-mediated protective effects on lung tissue damage following AP. To our knowledge, the present study is the first to demonstrate that

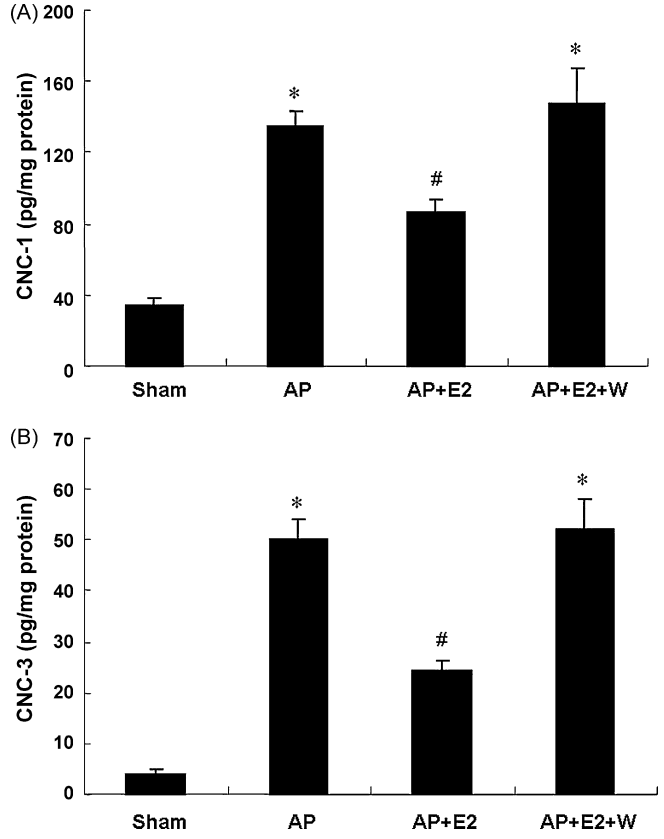

Fig. 5. Lung cytokine-induced neutrophil chemoattractant (CINC)-1 (A) and CINC-3 (B) levels at 8 h after sham or acute pancreatitis (AP). Animals were treated with vehicle, 17β-estradiol (E2), or E2 plus Wortmannin (W). Data are shown as mean ± SEM of 4–5 animals in each group. $^{*}p < 0.05$ vs. sham or trauma–haemorrhage–E2; $^{\#}p < 0.05$ vs. sham.

exogenous administration of E2 immediately after cerulean-induced AP significantly attenuates lung injury in a rodent model. Our results indicated that 8 h following AP, lung wet/dry weight ratios, MPO activity, IL-6, TNF-α, CINC-1 and CINC-3 levels were significantly increased. Administration of E2 1 h following AP markedly attenuated the above parameters. E2 treatment also prevented the AP-induced decrease in lung Akt phosphorylation. Administration of the PI3K/Akt inhibitor Wortmannin abolished the E2-mediated increase in Akt phosphorylation and decrease in pro-inflammatory mediator production following AP. These findings collectively suggest that a relationship seems to exist between E2 and Akt pathway in E2-mediated attenuation of lung injury following AP.

Neutrophils are part of innate immune response to infection, and thus, these cells have a protective effect.[6,20] However, under conditions such as AP, the tissue infiltration of neutrophils is increased.[6,19,36] Neutrophils also release superoxide anions and proteolytic enzymes, which diffuse across the endothelium and cause tissue damage.[20,31] The recruitment of neutrophil to sites of inflammation is also driven by locally produced cytokines and chemokines.[20,25,26,31] Studies have shown that IL-6 and TNF-α play important roles in the pathophysiology of AP-induced organ dysfunction and are required for expression of adhesion molecules and production of chemokines.[3,19] Moreover, it has been shown that treatment of rats with antibodies to neutralise the release of CINC-1 and CINC-3 in rat inflammation models decreases MPO buildup and neutrophil levels.[38]

There is increasing evidence that oestrogen is involved in regulating the posttraumatic pro-inflammatory mediator production.[4,14,32,40] Previous studies have also shown that E2 treatment following trauma–haemorrhage or ischemia reperfusion prevented the insult-induced increase levels of IL-6 and TNF-α in various tissues, including the lung.[27,34,42] In addition, CINC-1 and CINC-3, major cytokine-inducible neutrophil chemoattractants in rats, are essential for neutrophil emigration following injury such as AP or trauma–haemorrhage.[19,26] In the present study, our results indicated that E2 administration after AP attenuated the AP-induced increase in lung MPO activity and cytokine/chemokine production suggesting a role for E2 in the regulation of lung inflammation. In addition, similar to our previous findings that salutary effects of E2 on the heart and liver following trauma–haemorrhage are via activation of Akt,[25,28,29] our results suggested that the E2-mediated amelioration of lung tissue damage following AP was at least via normalisation of Akt phosphorylation.

Several studies have suggested that modulation of Akt phosphorylation by E2 is via the nongenomic pathway.[35,48] Furthermore, oestrogen receptors (ERs) such as ER-α, ER-β, and G-protein-coupled 30 have been reported to be involved in the E2-induced cellular signalling transduction after injury.[9,13,35,46] Studies have showed that reduced activation of PI3K and Akt was noted in female ER-β knockout hearts following ischemia reperfusion.[43] Our previous studies also have indicated that E2-mediated liver protection following trauma–haemorrhage was via an ER-related activation of Akt pathway.[25] Our current findings suggested that administration of E2 1 h after cerulein-induced AP restored the AP-induced decrease in lung Akt phosphorylation. Since in this study we measured lung Akt phosphorylation at 8 h after AP, the results lead us to speculate that the effects of E2 on the lung tissue are mediated via those receptors inducing cellular signalling transduction by the rapid response (nongenomic) pathway. Since lung Akt activation has been associated with protection of lung injury after AP and administration of E2 plus the Akt inhibitor Wortmannin immediately after AP attenuates lung Akt phosphorylation, it is reasonable to suggest that this hormone could potentially be used as a clinical adjunct to AP. Additional studies are, however, necessary to determine the safety profiles of this drug for clinical applications. In this regard, however, E2 (Premarin) is available and is approved for clinical use by the U.S. Food and Drug Administration. It should also be noted that we have administered only a single dose of E2, and thus, this is not to be considered as a hormone replacement therapy, which has received increased attention as being associated with an increased incidence of breast cancer and cardiovascular diseases.[1,37] Moreover, a population-based case–control study reported by Tetsche et al. did not support a substantial association between AP and the use of postmenopausal hormone replacement therapy (oestrogen or combined oestrogen/progestin).[41] Thus, we do not anticipate that a single dose of E2 would produce any deleterious effects in patients after AP.

It can be also argued that we should have administered E2 or Wortmannin alone in sham or Wortmannin in AP groups to determine if that, per se, has any adverse effects. In this regard, previous study has revealed that administration of E2 or Wortmannin alone in sham-operated or Wortmannin in trauma–haemorrhage groups did not produce any deleterious effects or influence organ function,[30,47] administration of E2 or Wortmannin alone was therefore not performed in this study. Furthermore, it can also be argued that whether administration of cerulein for 8 h in this study will induce AP and lung injury. Our previous studies have shown that 8 h after the experiment, lung oedema as well as pancreatic malnondialdehyde concentrations and water contents were significantly increased in cerulein-infused rats compared with those of sham-operated rats.[7]

Conclusions

Our results indicate that the E2 upregulates Akt phosphorylation and decreases lung oedema following AP. Blockade of Akt

phosphorylation and the associated deterioration of the examined parameters suggest that the reduction of neutrophils accumulation in the lung is in part mediated via Akt pathway. Thus, the Akt pathway seems to be required for E2-mediated attenuation of lung injury following AP.

Conflict of interest

None of the authors have any financial or other conflicts of interest to disclose.

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