**Abstract**

The present study aimed to assess several biomarkers: glucocorticoid receptor (GR), glucocorticoid (GC), and Phospholipase A2 (PLA2) in acute lung injury induced by fresh water drowning in partial adrenalectomized dogs. Twenty-four beagle dogs were randomly assigned into three equal groups: the sham control (Group C), the drowning group (Group D), and the drowning with partial adrenalectomy group (Group N). Animals in Group D and N were irrigated with 25 ml/kg fresh water into the right lung; the left lung without irrigation was set up as the concurrent control. Animals in Group C were used as the group control without irrigation. The GR in plasma and leukocytes, GC and PLA2 in plasma were compared between the two sides of lungs, as well as among different groups. GR in the right lung (RL) in Group D and N were rapidly lost reaching down to 64% of control level at 1h post irrigation, and to 85% at 8h. The loss in GR in LL was less pronounced; GRs were preserved at 72% of control level in Group N LL, which was significantly higher than in Group D LL (P<0.05). On the contrary, GR Kd rapidly increased. GR sites on the leukocytes in Group D and N were decreased over the time course. GC level in Group N was lower than in Group C, whereas GC level in Group D was quickly escalated till 4h post irrigation. PLA2 activities in Group D and N were escalated in an almost linear manner, where PLA2 in Group N was significantly higher than in Group D (P<0.01). It is concluded that there is rapid dynamic shifting of GR and GC which occurs immediately after drowning, particularly PLA2 that represents the process of inflammation in a real-time manner. This might provide some guidance for emergent critical care and medication.

**Keywords:** Lung, Drown, Glucocorticoid, Glucocorticoid Receptor, Phospholipase A2.
The pathogenesis of ALI/ARDS through the production of PLA2 has been identified in human, some of which are implicated in genes that encode various types of PLA2 proteins have activation catalytic mechanism. To date, at least 26 PLA2s are esterases that catalyze the hydrolysis of acyl groups at sn-2 position of glycerophospholipids (PL) and produce free fatty acids and lyso-PL by an interfacial acetylhydrolase could play important roles in the development or down-regulation of inflammation in ARDS, respectively (Nakos et al., 2005). Technically, Romaschin et al. found that PLA2 activity in arterial plasma but not in venous plasma was greatly elevated in parallel with ARDS (Romaschin et al., 1992). Kim et al. unraveled that PLA2 activity was significantly increased in broncho-alveolar lavage fluid (BALF), and may be more important as a biomarker than as one of general factors implicated in the pathophysiology of ARDS (Kim et al., 1995; Ortiz-Munoz et al., 2014; Liu et al., 2014).

Clinical symptoms of ARDS include refractory hypoxemia, noncardiogenic edema, and decreased lung compliance, so there is no perfect animal model to imitate and unravel the patho-physiological process of ARDS, because each animal model has its own significant benefits, but has its own weakness as well (Ballard-Croft et al., 2012). Our aim was to study whether GC and GR fluctuation in ARDS can pave the basis for clinical dexamethasone treatment, and particularly PLA2, whether it can be as a novel biomarker for future target. Though some drugs (e.g., dexamethasone) were applied to attenuate early seawater instillation-induced acute lung injury in rabbits, the basis for such a treatment was absent and left a gap to be filled.

### MATERIALS

[1,2,4- 3H ] Dexamethasone (3H Dex) was purchased from the Radiochemical Center (Amersham, England), 46 Ci/mM, the purity of 97% in stocking solution. Ten µCi/M of the stocking solution was diluted with 0.01 M Tris-HCl (pH7.4) as working solution. Phospholipase A2 (swine), Dexamethasone and Dextran T-700 were purchased from Sigma-Aldrich. ELISA radioactive kits for glucocorticoid measurement were purchased from Beijing North Institute of Biological Technology.

### Animal surgical methods

Beagle dogs were purchased from Experimental Animal Center, Nanjing University. Beagle dogs were about 6 months old, 8-10 kg body weight after two weeks acclimation. Each dog was identified with a unique number of ear tattoo, and individually housed in a steel single cage in our animal facility in accordance with AAALAC requirements.

All animals were tranquilized and anesthetized appropriately, and all surgical procedures were evaluated and approved by our Institutional Animal Care and Use Committee (IACUC). Total 24 beagle dogs (4 dogs/sex/group) were assigned into the sham control (Group C with sham surgery but without drowning), the drowning group (Group D with separation of the right and the left lungs isolated the primary bronchus, and the...
drowning with partial adrenalectomy group (Group N, isolated lungs drowning in partial adrenalectomized beagle dogs)

The partial adrenalectomy surgery and the canine drowning model were employed and slightly modulated according to previous studies, respectively (Larson et al., 2013; Conn et al., 1995; Fan et al., 2011; Xie et al., 2011; Han et al., 2012). Before the operation, dogs were overnight fasted, and were allowed to freely drink 10% chloral hydrate and gradually fell into sleep. Then, animals were intramuscularly injected with 20 mg/kg ketamine and 0.15 mg/kg atropine, and the appropriate status of anesthetizing was maintained with 0.1% thiopental sodium pentothal intravenous dripping during the operation.

The right adrenal gland between the right kidney and the inferior caval vein was resected. One-third of the left adrenal gland was also resected. The removed adrenals were identified by pathological diagnosis. The animals were analgestized with xylocaine 20 mg over the incision, and injected with prophylactic antibiotics (cefazolin, 20 mg/kg, Standard Chem and Pharm, Taiwan) on the following three days (Chu et al., 2013). Animals were allowed to fully recover for two weeks.

Tracheotomy was performed in all animals, (All the animals were conducted with tracheotomy), and then the left and right main bronchial tubes were separated with 37 size Carlen’s tracheal tube. Dogs in both the drown group, and the drown group with partial adrenalectomy were irrigated with 25 ml/kg of distilled deionized water (DDW) into the right primary bronchial tube over 5 min. Dogs in the sham control group underwent the same procedure with the exception of irrigation. Five ml heparinized (20 IU/L) blood was collected from each animal before the operation and at 0.5 h, 1 h, 2 h, 4 h, and 8 h post irrigation.

Analytical procedures

1. Determination of lung glucocorticoid receptor

Animals were euthanized with 50 mg/kg pentobarbital sodium. One gram lung tissue was taken out for homogenization with 4 times volume Tris buffer (pH 7.4) of the tissue weight (V/W). The homogenized lysate was first centrifuged at 4,000 X g for 15 min at 4 °C, and the supernatant was carefully transferred to other tubes, and then reloaded for super high speed centrifugation at 175,000 X g for 40 min at 4 °C. In the end, the final supernatant was taken out for measurement of the glucocorticoid receptors. The protein concentration was determined by Lowry’s method.

The glucocorticoid receptors (GR) in tissues were determined by using the radio-labeling ligand method (Nanni et al., 1982; Xu et al., 2005). Briefly, twenty mg protein lung tissue homogenized lysate was incubated with 10 μM ^3^H Dex solution (10 μCi[^3]^H) (The Radiochemical Center, Amersham, England) at 21 °C for 3 hours, and the reaction of binding was terminated with ten times volume of ice-cold 0.1M PBS (pH 7.4), and washed twice again with the same PBS. The protein was sedimented with 98% acetic acid, and washed three times with 15% acetic acid. Eventually the pellet was completely re-dissolved into 1 ml of 2.5 N NaOH and mixed with 3 ml scintillation liquid. The radioactivity (cpm) was determined on a LS 6500 Beckman Scintillation Counter. The non-specific binding was masked with 200 times of non-radio-labeled Dex for competitors. The blank control without lung tissue and the negative control with non-radio-labeled Dex solution were conducted in parallel. The regression equation was drawn from different concentrations of added[^3]^H Dex solutions, and plotted by Scatchard’s graph to calculate R₀ and Kd parameters.

2. Leukocyte suspension preparation

The leukocyte suspension from peripheral blood was prepared according to the method by Lapin et al., 1958). Five ml heparinized whole blood was diluted with twice volume of Hank’s Buffer, and mixed with half volume of 3% Dextran T500 gently and evenly. The tubes were allowed to stand in 4 °C refrigerator for 20 min. The upper layer of leukocytes was taken out and the remaining erythrocytes were quickly lysed with eight times volume of the lysing buffer (10 mM KPO₄, 155 mM NH₄Cl, 0.4 mM EDTA, pH 7.2), and incubated at 37 °C for 15 min, and then centrifuged at 1500 X g for 10 min. The pellets of leukocytes in the sediment were resuspended and washed with ice-cold PBS twice, and eventually were suspended as leukocytes/ml.

3. Determination of leukocyte glucocorticoid receptor

The suspension of leukocytes 0.5 ml (3 X 10^6 cells) was taken out to measure the amount of GR on leukocytes following the same procedure of determining GR in lung tissues but based on leukocyte numbers.

4. Determination of plasma glucocorticoid

The concentration of plasma glucocorticoid was determined by using radio-[^125]I-labeled immunoassay (Brock et al., 1978; Kumar et al., 1976). Briefly, 3 ml of the whole blood was collected with anti-coagulant heparin as before. The plasma was obtained by centrifugation at 1500 X g at 4°C for 15 min. All samples were snapped frozen in a liquid nitrogen tank and transferred to -80°C for storage until assay. The concentration of plasma glucocorticoid was calculated based on the standard curve.
5. Determination of plasma PLA2

The activity of PLA2 was determined according to literature (Reynolds et al., 1992). The principle underlines that PLA2 hydrolyzes the substrate of 2-Hio-Hexadecanoyl ethyl phosphocholine (HEPC) to yield the yellow color product of 5,5-dilhio-nifrobenzoic acid (DTNB), which can be read at 410 nm on a spectrophotometer. The absorbance is proportional to the activity of PLA2. Aliquots of plasma were taken from dogs by different treatments before drowning and at 1h and 8h of post drowning.

6. Statistical analysis

All the quantitative data were expressed as mean ± SD, and were subjected to statistical analysis by using One-way ANOVA program (SigmaStat 3.0; Systat Software Inc., Chicago, IL, USA). P value < 0.05 was considered to be statistically significant.

RESULTS

Comparison of dynamic alterations of GR in the different sides of lung tissue by different treatments

GR concentrations in the left lung tissue (LL) and the right lung tissue (RL) of dogs that had received different treatments were individually measured over the time course of drowning. As indicated in Table 1, higher levels of GR concentrations were detected in both sides of the lungs (the right and the left lungs) before drowning; however, after drowning 1h dogs in Group D, whose GR concentrations were decreased to about 37% in RL with irrigation, and about 50% in LL without irrigation, whereas dogs in Group N (after the partial adrenalectomy), whose GR concentrations were decreased to about 36% in RL with irrigation, and about 72% in LL without irrigation. Thus, such a decreasing trend went worse till 8h after drowning. GR concentrations in Group D dogs were decreased to about 16.8% in RL with irrigation, and about 31% in LL without irrigation, whereas GR concentrations in Group N dogs (after the partial adrenalectomy) were decreased to about 12.3% in RL with irrigation, and about 31% in LL without irrigation.

The GR Kd with Scatchard plotting analysis displayed similar profile. Kd, a constant factor of dissociation represents an opposite parameter of receptor affinity. As shown in Table 2, at 1h after drowning, GR Kd in RL with irrigation of Group D dogs was significantly increased as compared to that in RL of Group control dogs (P<0.01) but not in LL without irrigation of Group D dogs. The similar results were found in Group N dogs. At 8h after drowning, GR Kds in RL of both Group D and N were dramatically escalated as compared to those in Group C (P<0.01), but not found in LL without irrigation in both Group D and N. Nevertheless, GR Kds in RLs were significantly higher than those in LL in both Group D and Group N dogs at both 1h and 8h after irrigation (all P<0.05).

Comparison of dynamic alterations of GR on the surface of leukocytes in plasma under different treatments

GR concentrations on the surface of leukocytes were measured concurrently. As indicated in Figure 1, animals in Group D and N were bled before drowning and after drowning at 0.5h, 1h, 2h, 4h, and 8h, and animals in Group C (the sham control without irrigation) were bled at the same time points. GR concentrations on the surface of leukocytes in Group C (after the sham surgery) were slightly decreased over the time course, but the weight of difference only showed statistical significance at 4h and 8h. However, GR concentrations in both Group D and N were sharply decreased after 1h of post irrigation (P<0.01). At 2, 4 and 8h of post irrigation, the decreases of GR on the surface of leukocytes in both Group D and N were smooth and moderate, but the difference were still statistically significant (P<0.05) against the previous time point.

Starting from 2h post irrigation, the differences among three groups showed statistical significance. GR concentrations on the surface of leukocytes in both Group D and N were greatly decreased in comparison with those GRs in Group C at 2, 4 and 8h of post irrigation (P<0.01). However, the difference of GR concentrations on the surface of leukocytes displayed no statistical significance between GRs in Group D versus Group N.

Comparison of dynamic alterations of GC in plasma by different treatments

Apart from detection of GR expression on the surface of leukocytes in blood, GC concentrations in plasma were concurrently detected. After the sham surgery, the above GRs were slowly and gradually decreased in Group C dogs, but the contents of GC were gradually increased a little, which displayed in an opposite direction. Interestingly, the content of GC in plasma was flexible that greatly escalated from 0h to 4h, but plummeted to the original point at 8h of post irrigation in Group D dogs. Specifically, the levels of GC at 0.5h, 1h, 2h and 4h of post irrigation were higher than the level of GC before irrigation (0h) and the differences were of statistical significance. In contrast, the levels of GC in Group N dogs were relatively stable from 0h to 8h; nevertheless, GC contents in plasma in Group N dogs were about half amount of GCs in Group C dogs as compared at 0, 0.5, 1, 2, 4, and 8h of post irrigation, which evidently showed statistical significance (P<0.01, Figure 2).
Table 1. GR contents (fmol/mg protein) in lung tissues of both sides are compared among different groups of dogs by different treatments. The data were expressed as $\bar{x} \pm SD$ ($n = 8$). One way ANOVA followed by Dunnett’s test was employed statistical significance as $^B P<0.01$ when GR in Group D and N compared to GR in Group C, whereas $^a P < 0.05$ and $^b P < 0.01$ indicate GR in LL without DDW compared to GR in RL with DDW at the same time and in the same group (RL: right lung; LL: left lung).

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after drowning</th>
<th>0</th>
<th>1h</th>
<th>8h</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>RL</td>
<td>-</td>
<td>46.6±18.4$^a$</td>
<td>21.1±7.2$^a$</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>-</td>
<td>65.3±17.9$^b,a$</td>
<td>38.3±6.9$^b,a$</td>
</tr>
<tr>
<td>N</td>
<td>RL</td>
<td>-</td>
<td>44.7±14.2$^a$</td>
<td>15.4±8.3$^b$</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>-</td>
<td>90.3±16.8$^b,b$</td>
<td>39.1±5.8$^b,b$</td>
</tr>
<tr>
<td>C</td>
<td>RL/LL</td>
<td>125.2±13.9</td>
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</table>

Table 2. Alterations of GR Kd (nmol/L) are compared among different groups of dogs by different treatments. The data were expressed as $\bar{x} \pm SD$ ($n = 8$). One way ANOVA followed by Dunnett’s test was employed statistical significance as $^B P<0.01$ when GR Kd in Group D and N compared to GR in Group C, whereas $^a P < 0.05$, $^b P < 0.01$ indicates comparisons of GR within the same group (RL: right lung; LL: left lung).

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after drowning</th>
<th>0</th>
<th>1h</th>
<th>8h</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>RL</td>
<td>-</td>
<td>9.2±2.94$^a$</td>
<td>16.8±3.94$^a$</td>
</tr>
<tr>
<td></td>
<td>LL</td>
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<td>6.4±2.81$^a$</td>
<td>7.3±2.85$^a$</td>
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<td>9.6±3.12$^a$</td>
<td>15.8±3.63$^b$</td>
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<tr>
<td></td>
<td>LL</td>
<td>-</td>
<td>5.9±2.64$^a$</td>
<td>8.4±2.91$^b$</td>
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<tr>
<td>C</td>
<td>RL/LL</td>
<td>4.8±1.44</td>
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</tbody>
</table>

Figure 1. Dynamic alterations of GR (sites/cell) on the surface of leukocytes are compared among different groups of dogs by different treatments. The data were expressed as $\bar{x} \pm SD$ ($n = 8$). One way ANOVA followed by Dunnett’s test was employed statistical significance as $^B P<0.01$ when GR in Group D and N compared to GR in Group C at the same time point, whereas $^a P < 0.05$, $^b P < 0.01$ indicates comparisons of GR within the same group.
Figure 2. Dynamic alterations of plasma GC content (mg/L) are compared among different groups of dogs by different treatments. The data were expressed as $\bar{x} \pm SD$ (n = 8). One way ANOVA followed by Dunnett’s test was employed statistical significance as $^aP<0.01$, $^bP<0.05$ when GC in Group D and N compared to GC in Group C at the same time point, whereas $^aP<0.01$, $^bP<0.05$ indicates comparisons of GC within the same group.

Figure 3. Dynamic alterations of plasma PLA2 activity (IU/L) are compared among different groups of dogs by different treatments. The data were expressed as $\bar{x} \pm SD$ (n = 8). One way ANOVA followed by Dunnett’s test was employed statistical significance as $^aP<0.01$ when PLA2 in Group D and N compared to PLA2 in Group C and PLA2 in Group D compared to PLA2 in Group N at the same time point concurrently, whereas $^aP<0.01$ indicates comparisons of PLA2 within the same group.
Comparison of dynamic alterations of PLA2 activity by different treatments

The activity of PLA2 was measured before drowning and after drowning at 1h and 8h, respectively. The levels of PLA2 activities were rapidly escalated at 1h and 8h of post irrigation in Group N and D, and the differences at 1h and 8h as compared to 1h (before drowning) were statistically significant within the same group in both Group D and N dogs (P<0.01). Furthermore, the levels of PLA2 activities in both Group D and N over the time course (0—8h) were much higher than in Group C with statistical significance (P<0.01). The difference of PLA2 activities between Group D and N was so important that have to point out, as the levels of PLA2 in Group N were higher than those in Group D (P<0.01) at both 1h and 8h of post irrigation.

DISCUSSION

In the present study, the partial adrenalectomized canine model was initially reported in ALI/ARDS canine model. The key role of PLA2 activity, concomitantly with endogenous GR and GC were assessed in the development of ARDS induced by irrigation of fresh water to lungs. PLA2 activity is sharply escalated with the process of ARDS, but partial adrenalectomy slightly relieved the response of increased PLA2 activity. Although GC in drowned or partial adrenalectomized drowned dogs were rapidly increased, the increased GC could not serve its function because of lower endogenous GR, so it could not abolish the increase of PLA2. However, the partial adrenalectomy could preserve endogenous GR in order to alleviate the response of increased PLA2 activity. In addition, the right lung (RL) with irrigation and the left lung (LL) without irrigation display somehow different responses of GR and GR Kd where GRs were lost more in RL than in LL.

Since GR is expressed in almost every cell in the body, it has pleiotropic effects in different parts of the body, and can be classified into the following types of action:

Transactivation (Buckingham, 2006): Once the ligand binds to GR, homodimerization of GR are translocated from cytoplasm into the nucleus, binding to specific DNA responsive elements to activate gene transcription.

Transpression (Hayashi et al., 2004): Activated GR can complex with NF-κB, AP-1 etc. transcription factors and prevent them from binding their target genes, hence repress the expression of genes that are normally upregulated by NF-κB, AP-1.

The GR down regulation might be contributed to GC-mediated attenuation of gene expression and/or enhanced protein degradation via the proteasome (Rosewicz et al., 1988; Wallace and Cidlowski, 2001). On one hand, GR contents are quickly lost in drowning dogs, whereas GC levels immediately escalated, which exacerbates the decrease of GR through feedback. The lower levels of GR but not GC (as shown in our data) would release the restriction of inflammatory factors as PLA2, which aggravates the damage of ARDS; on the other hand, higher levels of GC induces neutrophilia from the marginal to the circulation pool (Nakagawa et al., 1998). Many leukocytes would penetrate and accumulate in the lungs by chemotactic cytokines, where these leukocytes release proteinase to disrupt lung tissues.

Dexamethasone is an agonist for GR. It has been reported that application of dexamethasone alleviates early seawater instillation-induced acute lung injury in rabbits (Xinmin et al., 2006).

In addition, GR contents on the surface of peripheral leukocytes are another contribution for us to monitor the decline of GR in the lung. Moreover, peripheral leukocytes play an important role in inflammation. Interestingly, the lower GC level in plasma induced by the partial adrenalectomy can greatly prevent from fluctuation after drowning, because upheaval of GC without correspondent GR would aggravate the damages of ARDS. As a consequence, the lower level of GR may help to release inflammatory factors, which leads to PLA2 activity skyrocketing in drowning dogs. The increased levels of PLA2 activity in the lung result in surfactant injury during ARDS, in part by changing surfactant composition through hydrolysis of phospholipids (Seeds et al., 2012).

PLA2, and other mediators as C5a, tumor growth factor (TGF-β), platelet activating factor (PAF), interleukins (IL-6, 10), tumor necrosis factor (TNF), are reported in the development of ALI/ARDS (Bosmann et al., 2014; Zhao et al., 2012; Ballard-Croft et al., 2012; Yang et al., 2014; Nakos et al., 2005; Kim et al., 1995; Anderson et al., 1994; Romaschin et al., 1992). PLA2 can disturb pulmonary functions either directly, by hydrolyzing lung surfactant phospholipids or indirectly, by stimulating or producing biologically active molecules, which leads to increased alveolar-capillary barrier permeability and lung edema formation. The prominent implication of secretory PLA2-IB and PLA2-IIA in inflammation and ALI/ARDS has been established long time ago in experimental models (Edelson et al., 1991; Sippola et al., 2006). PLA2 increased activity has been widely detected in BAL fluid, BAL cells and plasma, isoforms including type II A, secretary, cytolsic Ca-dependent, and Ca-independent forms (Nakos et al., 2005).

Intercepting PLA2 might be a good target to treat ARDS, because both PLA2-II expression and prostaglandin E2 (PGE2) synthesis can be completely suppressed after pretreatment of the cells with actinomycin D and cycloheximide, or dexamethasone (Kishino et al., 1994).

However, Our ARDS canine model was chosen based on previous studies. The partial adrenalectomy surgery was performed in dogs in this study to decrease the level
of glucocorticoid, and then the canine drowning model with fresh water was employed to study the role of glucocorticoid receptor in alleviating the damage of ARDS. It may not really reflect the drown status, and further study need to be done to explore a full spectrum mechanism in the future.

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REFERENCES


S biggest gap:


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