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Nicotinamide abrogates acute lung injury caused by ischemia-reperfusion

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Short Title:
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NICOTINAMIDE ON I/R LUNG INJURY

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ABSTRACT: Poly (ADP-ribose) synthase (PARS) or polymerase (PARP) is cytotoxic enzyme causing cellular damage. Nicotinamide, a compound of vitamin B complex, has been reported to exert inhibitory effect on PAPS or PARP. The present experiment tests the effects of nicotinamide on acute lung injury (ALI) and associated alterations following ischemia-reperfusion (I/R) of the isolated perfused rat's lung.

I/R increased lung weight (LW) to body weight ratio, LW gain, protein and dye tracer leakage, pulmonary arterial pressure, and capillary permeability. The insult also increased nitrate/nitrite, methyl guanidine, tumor necrosis factor$_{\alpha}$ and interleukin-1$_{\beta}$ in lung perfusate, while decreased adenosine triphosphate (ATP) content with an increase in PARP activity in lung tissue.

Most of the I/R-induced chagnes were abrogated by post-treatment (30 min after I/R) with nicotinamide (100 mg·kg^{-1} body weight). However, the increase in pulmonary arterial pressure was enhanced by nicotinamide post-treatment. Following I/R, the inducible nitric oxide synthase (iNOS) mRNA expression was enhanced. Nicotinamide reduced the iNOS expression.

The results suggest that nicotinamide exerted protective effect on the ALI caused by I/R. The mechanisms may be mediated through the inhibition on the PARP activity, iNOS expression and the subsequent suppression of nitric oxide, free radicals, and proinflammatory cytokines with restoration of ATP.

KEYWORDS: Free radical, nitric oxide, inducible nitric oxide synthase, poly (ADP-ribose) polymerase, proinflammatory cytokines

(INTRODUCTION)

Acute lung injury (ALI) following ischemia-reperfusion (I/R) has long been observed in various pulmonary disorders such as pulmonary arterial thromboendarterectomy [1], and lung transplantation [2]. Ischemia by a temporary interruption of blood flow to the lungs, followed by reperfusion causes lung injury evidenced by increases in lung weight, microvascular permeability, pulmonary arterial pressure and lung pathology [3, 4]. We have provided evidence to indicate the endogenous and exogenous nitric oxide (NO) are detrimental to the I/R lung injury [3], but static inflation exerts protective effects [4].

Nicotinamide is a compound of the soluble B complex. It exerts inhibitory effect on the poly (ADP-ribose) synthase (PARS) or poly (ADP-ribose) polymerase (PARP). The nuclear enzyme can be activated by strand breaks in DNA caused by reactive oxygen, nitrogen species, and peroxynitrite [5, 6]. PARP is cytotoxic by massive depletion of intracellular nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP). Inhibition of PARP activity reduces the ischemia-reperfusion injury of the heart, skeletal muscle and brain [6, 7]. In addition, PARP inhibition with 3-aminobenzamide attenuates the acute lung injury induced by endotoxin [8]. The inhibitory effects of nicotinamide or its related substances, niacinamide and nicotinic

acid on the PARP activity are protective to many organ dysfunction and/or cell damage caused by oxidative stress [9, 10]. Our laboratory has reported that PARS or PARP inhibition with niacinamide attenuates the ischemia-reperfusion hepatic injury [11].

Previous and recent studies from our laboratory have investigated the pathogenic mechanisms and potential therapeutic regimen on the acute lung injury (ALI) induced by endotoxin [12, 13], and ischemia-reperfusion [3, 4]. The present study was designed to evaluate the protective effect of nicotinamide on ALI caused by I/R in isolated perfused lungs. We also aimed to elucidate the possible mechanisms of the protective role of nicotinamide on the lung changes. Our results indicate that nicotinamide mitigates the ALI induced by ischemia-reperfusion.

METHODS

Experimental models

We used male rats of Spague-Dawley (SD) strain, 12-15 wk-old, weighing 330-360 g. The animals were obtained from the National Animal Center and housed in the University Laboratory Animal Center with adequate environmental control. The animal experiment was approved by the University Committee of Laboratory Animal Care and Use, and followed the guidelines of the National Animal Research Center. The room

temperature was maintained at 21±1 ℃ under a 12/12 h light/dark regimen. Food and water were provided *ad libitum*.

Acute experiments were carried out in isolated perfused rat's lungs. Rats were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (40 $mg \cdot kg^{-1}$). The rats were intubated with an endotracheal tube to provide ventilation of 95% room air-5% CO_2 with a rodent ventilator. The respiratory rate and tidal volume were 60-65 $breaths \cdot min^{-1}$ and 2-3 mL (6-9 $mL \cdot kg^{-1}$), respectively.

Isolation and perfusation of the lungs in situ

Isolated perfused lungs were prepared according to the procedures described in previous studies [3, 4]. A vertical incision was made along the midline of the thorax. Heparin (2 $IU \cdot g^{-1}$ body weight) was then injected into the right ventricle. An afferent silicon catheter was inserted into the pulmonary artery through the right ventricle. An efferent catheter was installed into the left atrium via the left ventricle. The pulmonary trunk and the aortae were tied. A third ligature was placed at the atrio-ventricular junction to prevent the perfusate from entering into the left ventricle. The isolated lungs were perfused with Krebs-Henseleit balanced solution with 6% albumin. The perfusion system included a venous reservoir and a roller pump. The pulmonary venous outflow

was diverted via the efferent catheter into the reservoir. The latter was placed in a 38 ℃ water bath for constant temperature. Pulmonary arterial pressure (PAP) and venous pressure (PVP) were measured from sideports in the afferent and efferent catheters. The lungs were perfused at constant flow (10-14 $mL \cdot min^{-1}$). Flow rate was adjusted to maintain the initial PAP at 15-16 mm Hg.

The isolated perfused lungs were left *in situ*, and the rat was placed on an electronic balance. The digital signals of the electronic balance were converted to analog signals by a digital-analog converter and recorded on a polygraph recorder. Weight changes were pre-calibrated on the electronic balance before preparation for the experiment. In this isolated lung *in situ* preparation, the changes in body weight (BW) reflected the lung weight (LW) changes [3, 4].

Lung weight (LW) and LW gain (LWG)

The initial LW was estimated from an equation relating to the body weight (BW) [12, 13].

$$LW\ (g) = 0.0015 \times BW\ (g) + 0.034$$

LWG was obtained from the increase in LW and also calculated as:

$$LWG = (\text{final LW} - \text{initial LW}) / \text{initial LW}$$

Protein concentration in bronchoalveolar lavage (PCBAL)

After the experiment, lungs were lavaged twice with saline (2.5 mL per lavage). Lavage samples were centrifuged at 1,500 g at room temperature for 10 min. The PCBAL was determined with a spectrophotometer by measuring the change in absorbance at 630 nm after the addition of bromocresol green [4].

Evans blue leakage

Five min prior to the end of experiment, Evans blue dye (1 $mg \cdot kg^{-1}$) was added into the lung perfusate. The dye concentration in lung tissue was determined spectometrically as described by Thurston et al. [14].

Microvascular permeability (K_{fc})

K_{fc} or capillary filtration coefficient as an index of microvascular permeability was calculated from the increase in LW produced by an elevation in PVP. The K_{fc} was defined as the initial weight gain rate ($g \cdot min^{-1}$) divided by PVP (10 cm H_2O) and LW, and expressed as $g \cdot min^{-1} \cdot cmH_2O^{-1} \cdot 100g^{-1}$. During the experiment, PVP was elevated rapidly by 10 cm H_2O for 7 min to measure K_{fc}. The hydrostatic challenge elicited a biphasic increase in LW: an initial rapid component followed by a slow and steady

component. The slow component of the weight gain was plotted on a semilog scale as a function of time. The capillary filtration rate was obtained by extrapolating the slow component of the weight gain back to zero time [3, 4].

Nitrate/nitrite, methyl guanidine, tumor necrosis factor$_\alpha$, and interleukin-1$_\beta$ in lung perfusate

Lung perfusate (0.5 mL) were taken 1 h before and 0.5, 1, 2, 3, 4, 5 and 6 h after ischemia/reperfusion (I/R). The samples were centrifuged at 3,000 g for 10 min. the supernatant was used for nitrate/nitrite measurement with high-performance liquid chromatography (ENO-20, AD Instruments, Mountain View, CA, USA) [15]. The formation of methyl guanidine (MG) has been identified as an index of hydroxyl radical production [16]. It was determined with its fluorescence spectrum (Jasco 821-FP, Spectroscopic CO., Tokyo, Japan). The emission maximum was set at 500 nm and the excitation maximum at 398 nm. The assay was calibrated with authentic MG (Sigma M0377). Tumor necrosis factor$_\alpha$ (TNF_α) and interleukin-1$_\beta$ ($IL\text{-}1_\beta$) were measured with antibody enzyme-linked immunosorbent assays (ELISAs) with a commercial antibody pair, recombinant standards, and a biotin-streptavidin-peroxidase detection system (Endogen, Rockford, IL, USA). All agents, samples and working standards were

prepared in room temperature according to the manufacturer's directions. The optical density was measured at 450/540 nm wavelengths by automated ELISA readers.

ATP content

Lungs were harvested after the experiments. A BioOrbit ATP Assay kit (Bio-Orbit Oy, Turku, Findland) was used to determine the lung ATP content with bioluminescence technique. The assay was based on quantitative measurement of a stable level of light as a result of an enzyme reaction catalyzed by luciferase. Under the effect of luciferase, the luminescence evoked by interaction of ATP and luciferin was recorded photometrically after amplification by a photomultiplier. The sensitivity of ATP was in nanomolar range. We used the luciferin-luciferase reagent according to manufacturer manual. ATP served as the standard. The test procedures were described previously [11].

PARP activity

PARP activity in the harvested lung tissue was measured with a commercially available assay kit (Genzyme Diagnostics, Cambridge, MA, USA). Lung tissue samples were placed on ice in 2 mL buffer containing 50 mM Tris·Cl (pH 8.0), 25 mM $MgCl_2$ and 0.1 mM phenylmethylsulfonyl fluoride. The samples were homogenized for 30 s and then

sonicated for 20 s using an ultrasonic homogenizer. The suspension was centrifuged at 3,000 g for 5 min at 4 ℃. Supernatant containing 20 *u*g protein, PARS buffer, 1 mM NAD, 2 *u*Ci ^{32}P- labeled NAD (1 *u*Ci·*u*L^{-1}) and distilled water was mixed in a microcentrifuge tube. The reaction was allowed to proceed at room temperature for 1 min and stopped by adding 900 *u*L of TCA. Enzyme activity was determined by measuring the incorporation of radiolabeled NAD as PARP catalyzed the poly (ADP) ribosylation of proteins. The labeled ADP was determined by scintillation counting after TCA precipitation onto a filter. The procedures and calculation of PARP activity were according to those described by Pulido et al. [17].

Inducible and endothelial NO synthases (iNOS and eNOS) mRNA

Reverse-transcriptase polymerase chain reaction (RT-PCR) was employed for a semiquantitative detection of iNOS and eNOS mRNA expression in the lung tissue. Total cellular RNA was extracted from each tissue section after the experiment by the Trizol procedure (Gibco BRL, Gaithesburg, MD, USA). Primers specific for iNOS (sense: 5'-CTTCAGGTATGC-GGTATTGG-3'; antisense: 5'-CATGGTGAACACG-TTCTTGG-3'), eNOS (sense: 5'-AGCTGGCATGGGCAACTTGAA-3'; antisense: 5'-CAGCACATCAAAGCGGCCATT-3'), and GAPDH (sense: 5'-TCCCTCAAGAT- TCTCAGCAA-3'; antisense:

AGATCCACAACGGATACATT-3') were used. Scanning densitometry was performed with Image Scan & Analysis System (Alpha-Innotech Corp., San Leandro, CA, USA). We followed the procedures described in relevant study [18] and manufacturer direction.

Ischemia-reperfusion (I/R)

Ischemia-reperfusion was produced in the isolated perfused lungs similar to the procedures described by Kao et al. [3, 4]. In brief, lungs were initially ventilated with 5% CO_2-95% N_2 for 10 min to decrease the O_2 content in the perfusate. Thereafter, ventilation and perfusion were stopped for 95 min. To facilitate subsequent reperfusion, the lungs were kept at an inflation state during the 95 min of ischemia by holding the ventilation at an inspiration state. The endotracheal pressure was set at 2 cm H_2O. After the ischemia, the lungs were reperfused and ventilated with 95% room air and 5% CO_2 for 50 min. The isolated perfused lungs were observed for 6 h after I/R.

Experimental protocols

Thirty isolated lung preparations were randomly subjected to vehicle, I/R with and without NCA post-treatment (n=10 in each of the three groups). In isolated perfused lungs, nicotinamide (100 mg·kg^{-1} body weight) was added into the venous reservoir.

We obtained lung weight changes, PCBAL, Evans blue content, K_{fc} and PAP. Nitrate/nitrite, MG, TNF_{α} and IL-1_{β} in lung perfusate, and ATP, PARP activity, NOS mRNA in lung tissue were determined. Nicotinamide was purchased from Sigma Chemicals (St. Louis, MO, USA). It was dissolved in physiological saline solution (PSS) before use. Vehicle groups received PSS. Nicotinamide (NCA) was given 30 min after 95 min ischemia and 50 min reperfusion. The dose of NCA administration into isolated lungs was in the range used in previous studies [11, 17, 19-21].

Data analysis

Data were expressed as means ± SEM. Comparisons within and among groups were made with one-way analysis of variance with repeated measures followed by *post hoc* comparison with Newman-Keul's test. A P value less than 0.05 was considered statistically significant.

RESULTS

Lung weight changes, pulmonary arterial pressure, and microvascular permeability (K_{fc})

In isolated lungs, I/R caused increases in lung weight (LW)/body weight (BW) ratio, lung weight gain (LWG), and K_{fc}. The changes in lung weight and capillary filtration

were reduced by NCA post-treatment. I/R increased the pulmonary arterial pressure. Post-treatment with NCA augmented the I/R-induced pulmonary hypertension (fig. 1).

Protein concentration in bronchoalveolar lavage (PCBAL) and Evans blue dye content

I/R caused increases in protein and tracer dye leakage in the isolated lungs. Post-treatment with NCA attenuated the PCBAL and tracer dye content (fig. 2).

ATP content and PARP activity in lung tissue

I/R resulted in decrease in ATP content and increase in PARP activity. NCA post-treatment reduced the I/R-induced elevation of PARP, while restored the ATP content (fig. 3).

Nitrate/nitrite, methyl guanidine, tumor necrosis factor$_{\alpha}$, and interleukin-1$_{\beta}$ in lung perfusate

The NO metabolites, hydroxyl radical and proinflammatory cytokines in lung perfusate were increased remarkably following I/R. NCA post-treatment abrogated the I/R-induced changes (fig. 4).

Expression of inducible and endothelial nitric oxide synthases (iNOS and eNOS) mRNA in lung tissue

Fig. 5 shows the expression of iNOS and eNOS mRNA in lung tissue following vehicle, I/R and I/R with NCA. Table 1 summarizes the values of iNOS/GAPDH and eNOS/GAPDH ratio. The data indicated that I/R in isolated lungs caused a great increase in iNOS mRNA expression and a slight increase in eNOS mRNA expression. These effects were diminished by post-treatment with NCA.

DISCUSSION

In the present study, we employed ischemia-reperfusion to induce acute lung injury in isolated perfused rat's lungs. In our previous study, static lung inflation was able to reduce the I/R lung injury [4]. In the present study, the isolated lung was kept in an inspiration state. The differences in the experimental design were that the length of ischemia was 95 min and the endotradeal pressure was 2 cm H_2O compared to 75 min ischemia and an endotracheal pressure at 5 cm H_2O in that previous study. The prolongation of ischemia period and reduction in endotracheal pressure caused different extent of I/R lung injury.

We found that nicotinamide, a PARP inhibitor exerted protective effects on the ALI following I/R. This agent reduced the changes in lung weight, microvascular permeability, protein and dye leakage. In addition, NCA resulted in reduction of NO metabolites, hydroxyl radical and proinflammatory cytokines in the lung perfusate after I/R. I/R enhanced PARP activity, NOS mRNA expression (iNOS, in particular) and decreased ATP content. Nicotinamide, the vitamin B related agent reduced the PARP activity and iNOS mRNA expression and restored ATP. A previous study from our laboratory has revealed the beneficial actions of NCA on the I/R hepatic injury [11]. We further demonstrated the protective role of NCA on the ALI induced by I/R.

Pharmacological inhibition and gene knockout mutant of poly (ADP-ribose) synthase (PARS) or poly(ADP-ribose) polymerase (PARP) have become a new approach for the experimental therapy of various disorders such as endotoxin shock, stroke, ischemia/reperfusion injury, lung inflammation and others [6, 7, 17, 20-23]. Activation of PARS or PARP produces cytotoxicity and subsequent cell death and organ dysfunction [24]. We demonstrated in the present study that post-treatment with nicotinamide abrogated the ALI induced by ischemia-reperfusion in isolated lungs.

A review article has addressed that various pharmacological stimulations are able to facilitate alveolar epithelial fluid transport and thereby reducing the acute lung injury caused by different challenges [25]. Whether nicotinamide affects alveolar epithelial water and ion transport system requires further investigation.

In the lungs, PARS or PARP plays key role in the microvascular platelet-endothelial cell interaction induced by endotoxin, acute lung inflammation following intratracheal administration of endotoxin, induction of asthma and leukocyte recruitment in systemic endotoxemia [8, 21-23]. These cellular interactions, tissue inflammatory changes and the associated changes in adhesion molecules are likely the fundamental basis for the pathogenesis of lung injury. In the present investigation, we revealed that I/R increased the PARP activity, while reduced the ATP content. NCA attenuated the I/R-induced increase in PARP activity and restored the ATP content. The protective effect of NCA may be mediated at least in part through the inhibition of PARP and restoration of ATP.

In addition to the role of PAPS or PARP in the acute lung injury due to various causes, iNOS may also be a crucial factor in the lung damage. In endotoxin-induced lung damage, many studies including those from our laboratory have provided evidence to suggest that NO production through the iNOS is harmful to the lungs in various species

and causes [26, 27]. In patients with lung inflammation or injury, the iNOS expression and nitrotyrosine activity were increased [28]. PARP was involved in NFκB expression and thereby activated NOS. ADP-ribosylation is required to activate NFκB-mediated iNOS gene transcription [29]. Remick et al. [30] also suggested that ADP ribosylation was crucial in the signaling pathway which leads to NOS mRNA expression. PARP inhibitors prevent NOS induction, iNOS mRNA expression and TNF_{α} release in interferon and LPS stimulated macrophages. After inhibition of PARP, iNOS expression, iNOS activity and NO production were reduced [8]. PARP inhibition also reduced the production of peroxynitrite and prevented the presence of nitrotyrosine in the tissue [19]. It is likely that iNOS is involved in the pathogenesis of tissue injury following endotoxemia, ischemia-reperfusion and other challenges in various organs. In the present study, we used RT-PCR to demonstrate that I/R upregulated iNOS expression. The upregulation was attenuated by post-treatment with NCA.

In the present study, we found that pulmonary arterial pressure was elevated by I/R and post-treatment with NCA augmented the I/R-induced pulmonary hypertension. The finding may imply that NCA acts as an inhibitor of iNOS, and the reduction in NO release may account for the effect of NCA on the I/R-induced pulmonary hypertension. In this regard, the pulmonary vasodilatory effect of NO may be the sole benefit in I/R

lung injury. It is noteworthy that the increase in microvascular permeability was not associated with the changes in pulmonary arterial pressure.

In conclusion, nicotinamide protects the lungs from injury by I/R. The mechanisms are possibly mediated through the inhibitory effects of this agent on the PARP activity and iNOS expression. Subsequently, the production of NO, free radical and proinflammatory cytokines was suppressed, while the ATP content was restored. Since nicotinamide is a compound of vitamin B complex, the protective effects of nicotinamide on the I/R lung injury may be a potential therapeutic or preventive regimen for clinical application in lung transplantation or other conditions of lung ischemia and reperfusion.

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Figure Legend

FIGURE 1. The maximal values of lung weight (LW) to body weight (BW) ratio (LW/BW, a), LW gain (LWG, b), microvascular permeability (K_{fc}, c) and pulmonary arterial pressure (PAP, d) in isolated lungs subjected to I/R with and without nicotinamide (NCA) post-treatment. I/R increased the lung weight change. The increases were abrogated by NCA post-treatment. I/R challenge elevated the PAP, and the I/R-induced pulmonary hypertension was further augmented by NCA post-treatment. *$P < 0.05$ vs. Vehicle; $^{+}P < 0.05$ vs. I/R.

FIGURE 2. Protein concentration in bronchoalveolar lavage (PCBAL, a), Evans blue content in lung tissue (b). I/R increased the PCBAL and tracer dye leakage. Post-treatment with NCA abrogated the I/R-induced protein and dye permeation. *$P < 0.05$ vs. vehicle; $^{+}P < 0.05$ vs. I/R.

FIGURE 3. Adenosine triphosphate (ATP) content (a) and poly (ADP-ribose) polymerase (PARP) activity (b) in lung tissue. I/R decreased the ATP content, while increased the PARP activity. Post-treatment with NCA reduced the PARP activity, while restored the ATP content. *$P < 0.05$ vs. vehicle; $^{+}P < 0.05$ vs. I/R.

FIGURE 4. Nitrate/nitrite (a), methyl guanidine (b), tumor necrosis factor$_{\alpha}$ (c) and interleukin-1$_{\beta}$ (d) in lung perfusate. I/R significantly elevated these factors. Post-treatment with NCA attenuated the effects of I/R on the nitric oxide metabolites, hydroxyl radical and proinflammatory cytokines. *P < 0.05 vs. vehicle group; ^{+}P < 0.05 vs. I/R group.

FIGURE 5. Expression of inducible and endothelial NO synthase (iNOS and eNOS) mRNA with reverse-transcriptase polymerase chain reaction in the lung tissues following various treatments. The expression of GAPDH was used as an internal standard.

FIGURE 1

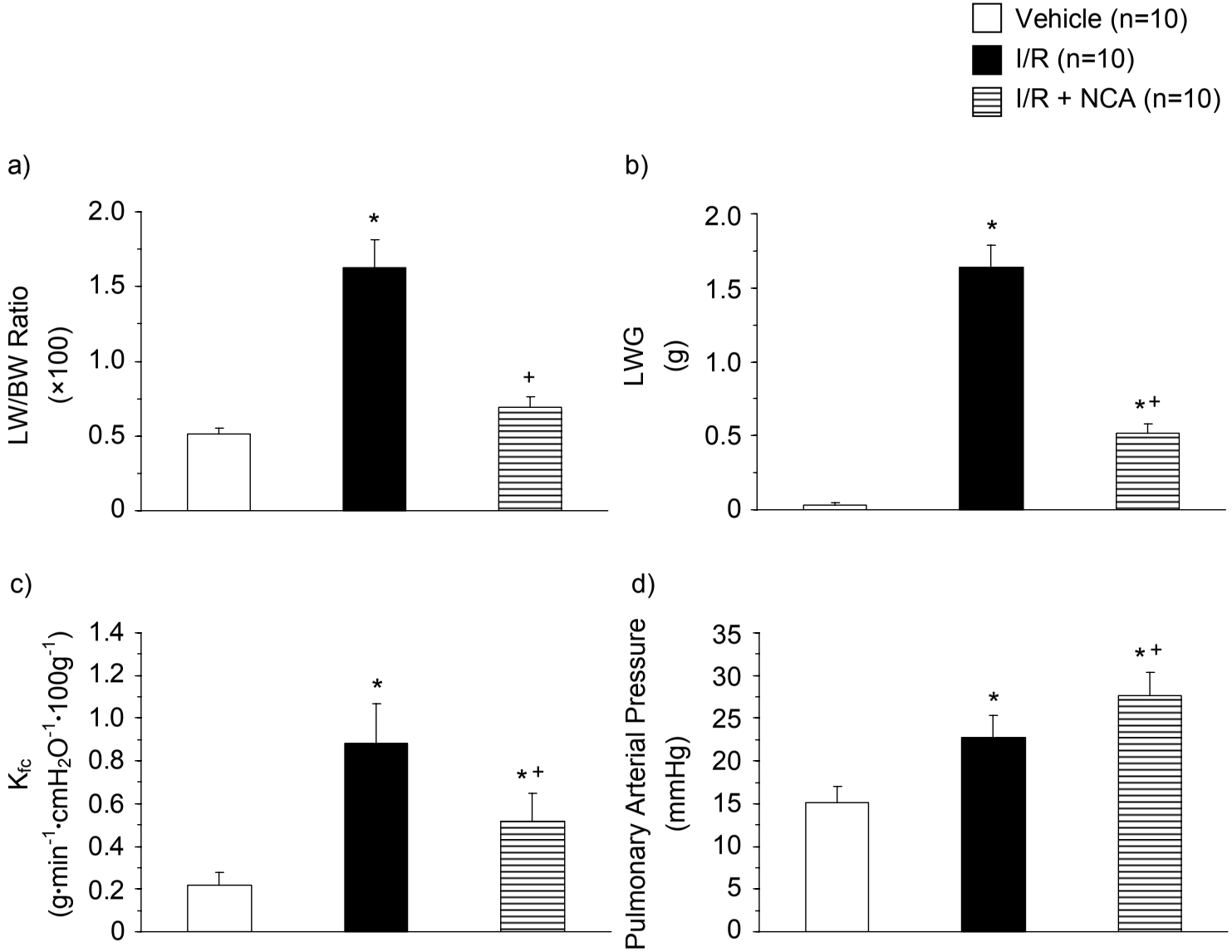
Vehicle (n=10)
I/R (n=10)
I/R + NCA (n=10)
a)
LW/BW Ratio (×100)
2.0
1.5
1.0
0.5
0
*
+
b)
LWG (g)
2.0
1.5
1.0
0.5
0
*
*+
c)
K_{fc} ($g \cdot min^{-1} \cdot cmH_2O^{-1} \cdot 100g^{-1}$)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*+
d)
Pulmonary Arterial Pressure (mmHg)
35
30
25
20
15
10
5
0
*
*+

FIGURE 2

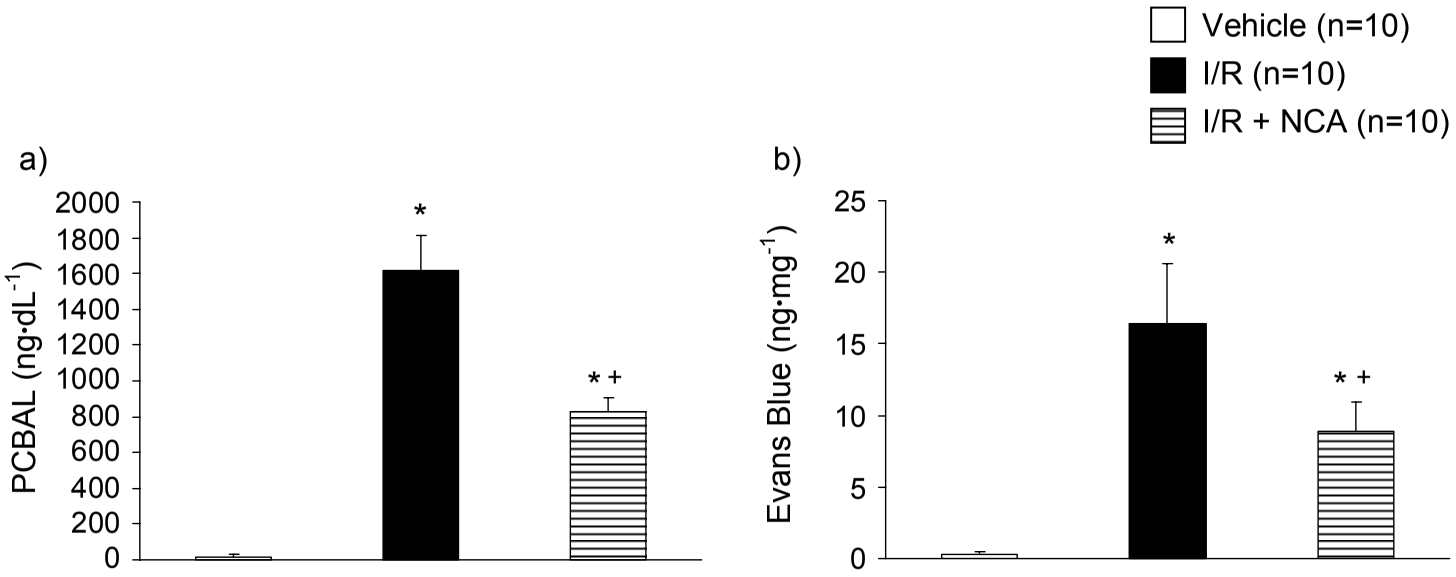
Vehicle (n=10)
I/R (n=10)
I/R + NCA (n=10)
a)
PCBAL (ng·dL-1)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
* +
b)
Evans Blue (ng·mg-1)
25
20
15
10
5
0
*
* +

FIGURE 3

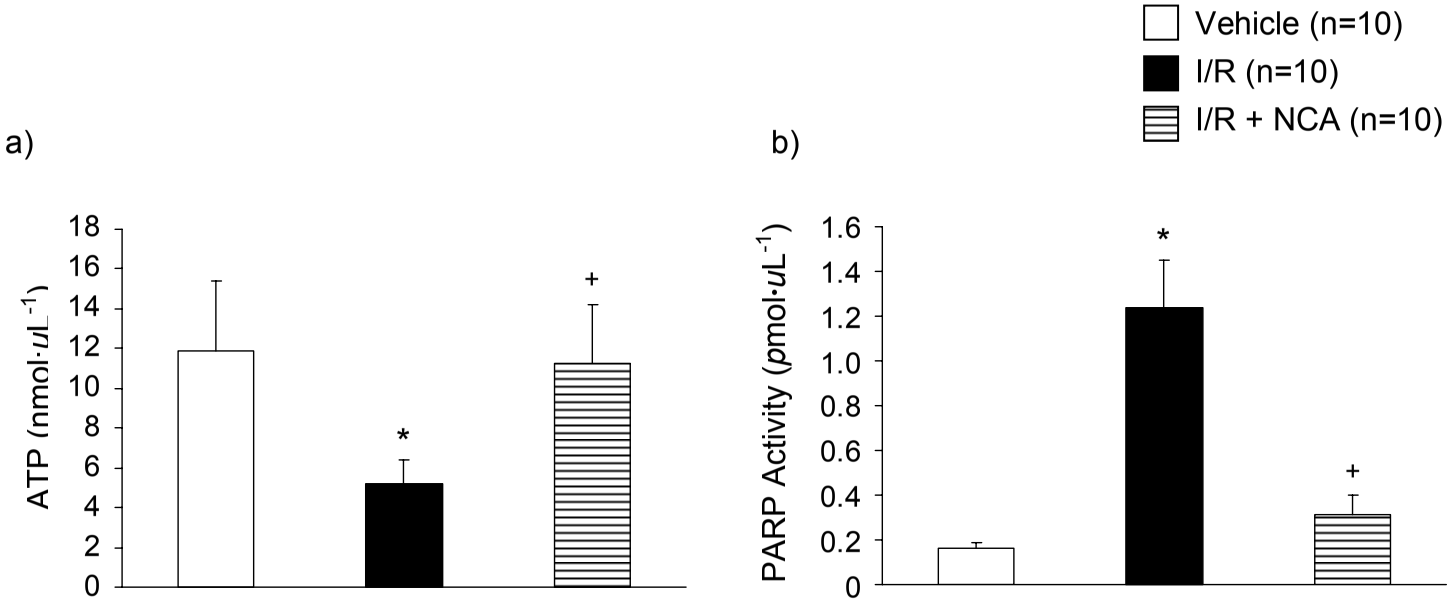
Vehicle (n=10)
I/R (n=10)
I/R + NCA (n=10)
a)
b)
ATP (nmol·uL-1)
PARP Activity (pmol·uL-1)
18
16
14
12
10
8
6
4
2
0
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
+
*
+

FIGURE 4

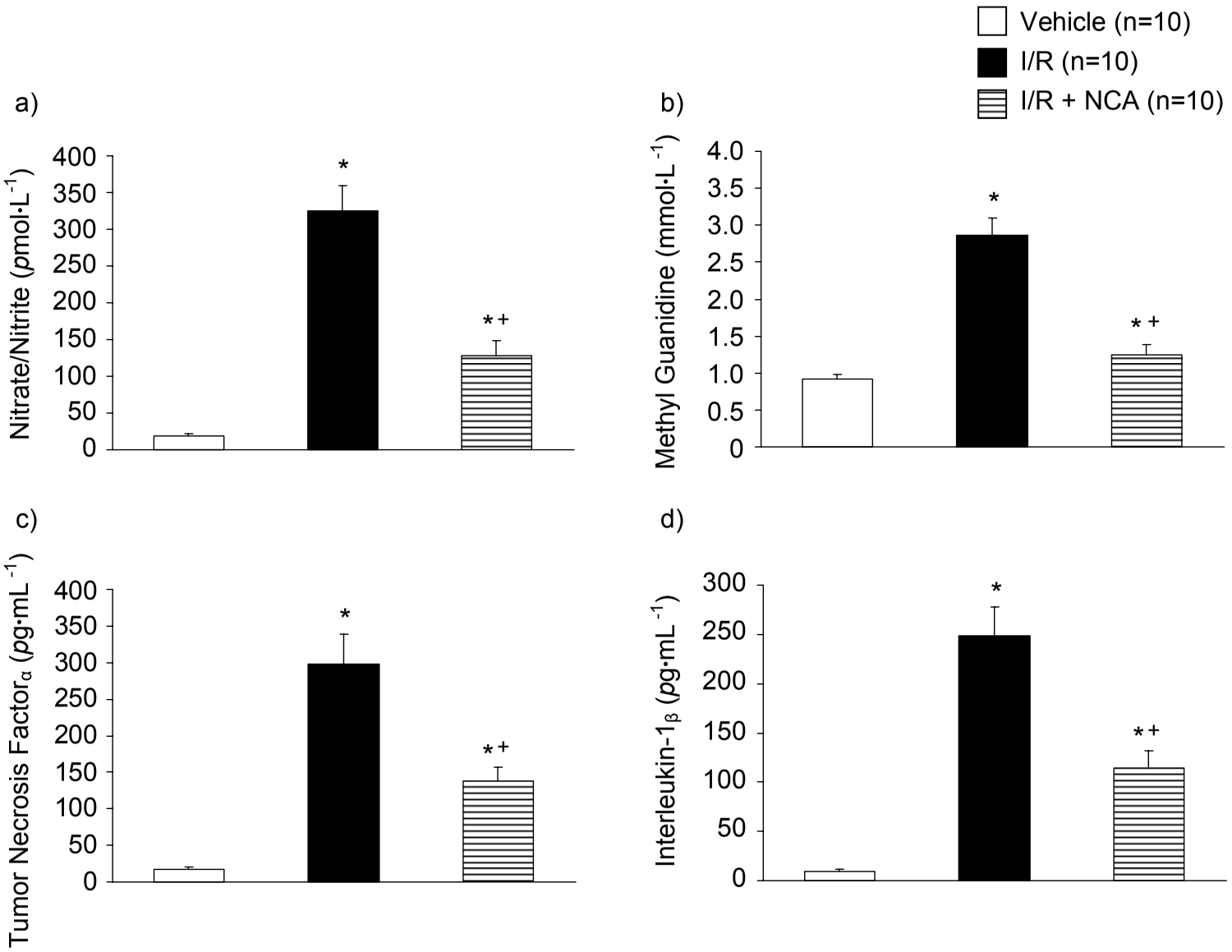
Vehicle (n=10)
I/R (n=10)
I/R + NCA (n=10)
a)
Nitrate/Nitrite (pmol·L-1)
400
350
300
250
200
150
100
50
0
*
*+
b)
Methyl Guanidine (mmol·L-1)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*+
c)
Tumor Necrosis Factorα (pg·mL-1)
400
350
300
250
200
150
100
50
0
*
*+
d)
Interleukin-1β (pg·mL-1)
300
250
200
150
100
50
0
*
*+

FIGURE 5

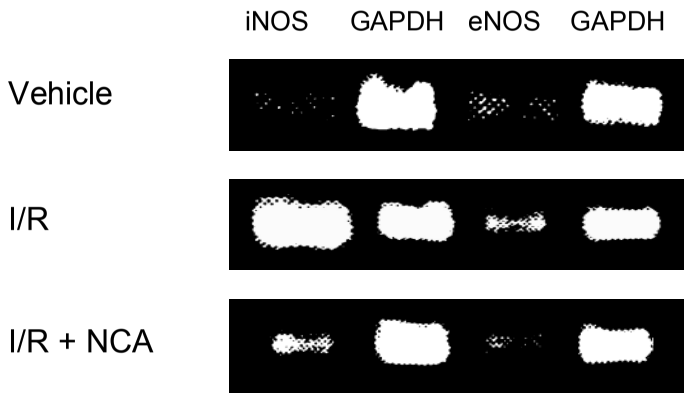

TABLE 1 Inducible and endothelial nitric oxide synthases (iNOS and eNOS) mRNA expression in lung tissue

	iNOS/GAPDH ratio	eNOS/GAPDH ratio
Vehicle	0.04 ± 0.02	0.15 ± 0.05
I/R	1.64 ± 0.13*	0.53 ± 0.06*
I/R + NCA	0.72 ± 0.06*$^{+}$	0.06 ± 0.03*$^{+}$

Values are mean ± SEM (n=10 in each group). *P < 0.05 compared with the value in vehicle group; ^{+}P < 0.05 compared with the value in I/R group.