

Increased Monoamine Oxidase Activity of Lung with Ischemia-Reperfusion Injury: Effect of Preconditioning

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Summary

Recent studies have been focused on the protective role of ischemic preconditioning (IP) against ischemia reperfusion (I/R) injury of the lung occurring following cardiopulmonary bypass or lung transplantation. Although reactive oxygen species (ROS) production has been postulated to play a crucial role in I/R, the sources of ROS during I/R are still unclear. Since it has been previously described that monoamine oxidases (MAOs) are a potential source of hydrogen peroxide (H₂O₂) generation in early reperfusion following ischemia, the present study aimed to investigate the possible contribution of MAO to ROS generation and lipid peroxidation during I/R and IP protocols in the lung. Male Wistar rats were randomly divided into three groups: control lungs were subjected to 30 min. of perfusion; lungs of the I/R group were subjected to 2 h of cold ischemia following 30 min. of perfusion; and in the third group IP was performed by two cycles of 5 min. ischemia followed by 5 min. of reperfusion prior to 2 h of cold ischemia and then reperfusion. MAO-A and B activities, lipid peroxidation, reduced (GSH) and oxidized (GSSG) glutathione levels, H₂O₂ release and catalase activity were determined in tissue samples. MAO-A and B activities, lipid peroxidation, GSSG content and H₂O₂ release were found to be increased, while GSH content, GSH/GSSG ratio and catalase activity were decreased in lung tissues of the I/R group when compared with those of the control group. MAO-A and B activities, lipid peroxidation, GSSG content and H₂O₂ release were found to be decreased, while GSH content, GSH/GSSG ratio and catalase activity were increased in lung tissues of the IP group when compared with those of the I/R group. Strong positive correlations were found

İskemi-Reperfüzyon Hasarlı Akciğerde Artmış Monoamin Oksidaz Aktivitesi: Önkoşullamanın Etkisi

Özet

Son yıllarda yapılan çalışmalar, akciğerdeki iskemik önkoşullamanın (IP) kardiyopulmoner by-pass veya akciğer transplantasyonu sonrasında oluşan iskemik reperfüzyon (I/R) hasarının önlenmesindeki koruyucu rolü üzerinde yoğunlaşmaktadır. İskemik önkoşullamada reaktif oksijen türlerinin (ROS) önemli rol oynadığı gösterilmekle birlikte, oluşan radikallerin kaynağı açıklık kazanmamıştır. Daha önce yapılan çalışmalarda monoamin oksidaz (MAO) enziminin, iskemiye takip eden erken reperfüzyon esnasında oluşan hidrojen peroksit (H₂O₂) 'in kaynağı olduğu öne sürüldüğünden, bu çalışmada MAO 'ın akciğerde I/R ve IP esnasında ortaya çıkan ROS 'a katkısının araştırılması amaçlandı. Üç gruba ayrılan erkek sıçanların kontrol grubunu oluşturanları 30 dakikalık perfüzyona tabi tutuldu; I/R grubunda 30 dakikalık perfüzyonu takiben 2 saatlik soğuk iskemi oluşturuldu ve IP grubunda IP, 2 saatlik soğuk iskemi öncesi iki döngü şeklinde uygulanan 5 dakikalık iskemi, 5 dakikalık reperfüzyon ile sağlandı. Doku örneklerinde MAO-A ve B aktiviteleri, lipid peroksidasyonu, redükte (GSH) ve okside glutatyon (GSSG) düzeyleri, H₂O₂ oluşumu ve katalaz aktivitesi tayin edildi. I/R grubunda MAO-A ve B aktiviteleri, lipid peroksidasyonu, GSSG içeriği ve H₂O₂ salınımı artmış; GSH içeriği, GSH/GSSG oranı ve katalaz aktivitesi azalmış olarak bulundu. IP grubunda MAO A ve B aktiviteleri, lipid peroksidasyonu, GSSG içeriği ve H₂O₂ salınımı azalmış; GSH içeriği, GSH/GSSG oranı ve katalaz aktivitesi artmış olarak tesbit edildi. I/R ve IP gruplarında MAO aktivitesi ile H₂O₂ oluşumu arasında kuvvetli pozitif bir ilişki bulundu ve MAO enziminin reperfüzyon esnasında oluşan fazla H₂O₂'in kaynağı olduğu ve

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between MAO activity and H_2O_2 release in lung tissues of I/R and IP groups, suggesting that MAO is a potential source of H_2O_2 generation during reperfusion and that IP protects the lung against oxidative damage via diminishing MAO-mediated excess H_2O_2 formation. Although the present study is preliminary by design, we suggest that MAO isoforms may contribute to ROS generation during I/R, and that MAO inhibitors may be used together with IP to protect against lung injury during I/R.

Key Words: Preconditioning, ischemia-reperfusion, monoamine oxidase.

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IP'nin MAO-kaynaklı H_2O_2 oluşumunu azaltarak akciğer dokusunu I/R hasarına karşı koruduğu öne sürüldü. Her ne kadar bu çalışma bir öncül araştırma niteliğinde ise de, MAO izoformlarının I/R esnasında oluşan ROS'ne katkıda bulunduğu ve MAO inhibitörlerinin IP ile birlikte uygulanmasının iskemik akciğer hasarının önlenmesinde kullanılabileceği kanısına varıldı.

Anahtar Kelimeler: Önkoşullama, iskemi-reperfüzyon, monoamin oksidaz

INTRODUCTION

Ischemia-reperfusion (I/R) injury, a complex phenomenon often seen in surgical practice, such as in pulmonary embolism, cardiopulmonary bypass, or lung transplantation, is associated with both local injury and induction of systemic inflammatory response¹⁻⁴. I/R injury has also been attributed to endothelial damage resulting in an increased permeability and resistance in the pulmonary vascular system^{5,6} and is related to a number of factors, such as energy degradation during ischemia, generation of reactive oxygen species (ROS) during reperfusion, "no-reflow" phenomenon, and calcium overload reperfusion⁷. Ischemic preconditioning (IP), an adaptive pathophysiological condition which is defined as brief and repetitive episodes of ischemia-reperfusion before a sustained IR, renders the lung more tolerant to subsequent sustained I/R injury^{8,9}. Although the mechanisms of IP are not fully elucidated, it has been recently proposed that ROS excessively formed during IR may cause lipid peroxidation of cell membranes, protein and enzyme oxidation and some irreversible DNA changes, leading to cell death and contributing to I/R^{10,12}. Much research has been focused on identifying sources of ROS and determining whether increased oxidant production is a component of I/R injury. However, the sources of ROS and the natural protective mechanisms against excess ROS generation in I/R are still controversial.

Monoamine oxidase (MAO, EC 1.4.3.4) is a flavoenzyme which plays an essential role in the oxidative deamination of biogenic amines such as serotonin, adrenaline, noradrenaline and dopamine, both in the central nervous system and in peripheral tissues¹³; it also catalyzes the oxidation of xenobiotic amines¹⁴. MAO is found in two different forms, designated as MAO-A and MAO-B, which are encoded by two different genes¹⁵ and distinguished by different substrate specificities and sensitivities to the selective inhibitors^{16,17}. MAO-A preferentially oxidizes serotonin and noradrenaline and is irreversibly inhibited by clorgyline, while MAO-B preferentially oxidizes benzylamine and phenylethylamine and is reversibly inactivated by pargyline. Dopamine, tyramine and tryptamine are reported as common substrates for both MAO forms^{18,19}. It has been shown that MAO catalyzes the oxidative deamination of biogenic amines to their corresponding aldehydes. This is accompanied by the reduction of molecular oxygen to hydrogen peroxide H_2O_2 , which cannot be fully scavenged by endogenous antioxidants²⁰. MAOs also contribute to increase in H_2O_2 production during renal I/R²¹. The toxicity of H_2O_2 is suggested to originate from its ability to induce oxidative damage to the proteins directly as H_2O_2 or through its conversion into hydroxyl radicals via Fenton reaction²². It was postulated that intramitochondrial hydroxyl radicals from H_2O_2 gene-

rated during MAO metabolism serves as a major contributor to tissue injury in the brain²³.

The purpose of the present study was to expand on these findings to further characterize the contribution of MAO activation and MAO-mediated H₂O₂ production to oxidative damage occurring during I/R in the lung, and to investigate the possible protective effect of IP against this phenomenon.

MATERIALS and METHODS

Chemicals

All chemicals were obtained from Sigma Chemical Co. (Germany). Heparin (Nevparin) and thiopental sodium (Pental sodium) were kindly provided by Mustafa Nevzat Pharmaceuticals, Istanbul, and Ibrahim Ethem Pharmaceuticals, Istanbul, respectively.

Isolated lung preparation

The animal experimentations were approved by the Ethics Committee of Laboratory Animals, Hacettepe University, Turkey (# 2001/25-4). Male Wistar rats (200-300g) were anesthetized with thiopental (30 mg/kg, i.p.). After tracheal cannulation, the chest was opened and heparin (200 IU) was injected into the right ventricle. The main pulmonary artery was cannulated via the right ventricle and the vasculature was flushed with Krebs-Henseleit solution [(KHS, in mM): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 25, MgSO₄ 1.2, glucose 10]. The left atrium was cut and the major part of the ventricles removed to allow free afflux of the perfusate. The lung was removed, suspended in a chamber and perfused with KHS (bubbled with 95% O₂ and 5% CO₂ at 37°C) at a constant flow rate (0.03 ml.g⁻¹) by a peristaltic pump (Gilson Model M312). To inhibit the cyclooxygenase pathway, indomethacin (3 μM) was added to the perfusion solution. Mean perfusion pressure (PP) was measured via a pressure transducer attached to a side arm of the pulmonary artery cannula. Changes in PP were recorded on a computer-

based data acquisition system (TDA96). In the I/R group, after the 30 min. of constant flow perfusion, the lungs were subjected to 2 h hypothermic ischemia at 4°C in KHS. In the IP group, IP was performed by two successive cycles of 5 min. ischemia, followed by 5 min. reperfusion prior to the 2 h hypothermic ischemia (Fig. 1).

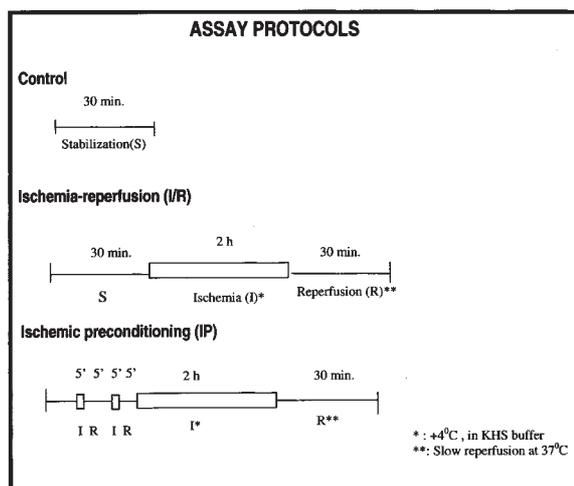


Figure 1. Experimental protocols

At the end of the protocols, the lungs were re-attached to the perfusion system, perfusion flow was gradually increased, and the same flow rate as prior to ischemic protocol was achieved within 10 min. Lungs were excised, weighed and the tissue portions were homogenized in 50 mM potassium phosphate (KP) buffer, pH 7.4.

Determination of malondialdehyde (MDA)

Lipid peroxidation in lung tissues was determined by the measurement of MDA levels on the basis of MDA reacted with thiobarbituric acid (TBA) at 532 nm, according to a previous method²⁴. The principle of the method was based on the spectrophotometric measurement of the colored complex generated by the reaction of TBA with MDA. MDA concentration was calculated using the molar extinction coefficient of the MDA-TBA complex, $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. Values were expressed as nmol. mg⁻¹.

Determination of reduced (GSH) and oxidized (GSSG) glutathione

GSH and GSSG were determined in the lung according to the method described previously²⁵. Total glutathione was determined using a kinetic assay in which amounts of GSH or GSSG and glutathione reductase brought about the continuous reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by NADPH. The formation of 5-thio-2-nitrobenzoate (TNB) was followed spectrophotometrically at 412 nm at 25°C. Total glutathione and GSSG were expressed as $\mu\text{mol. mg protein}^{-1}$. GSH was calculated as $[\text{total glutathione}] - 2 \times [\text{GSSG}]$ and expressed as $\mu\text{mol. mg}^{-1}$.

Determination of catalase (CAT) activity

CAT activity in lung tissue was determined according to the method of Ueda²⁶. Decomposition of H_2O_2 was monitored for 15 min. with a decrease in absorbance at 240 nm. A molar extinction coefficient of $43.6 \text{ M}^{-1}\text{cm}^{-1}$ was used to determine the activity. Enzyme activity was expressed as nmol. mg^{-1} .

Purification of mitochondrial MAO from rat lung homogenates

Mitochondrial MAO was purified by isolation of mitochondria from lung homogenates²⁷. Lung tissue (5-8 g) was homogenized 1:40 (w/v) in 0.3 M sucrose. Following centrifugation at $1,000 \times g$ for 10 min., the supernatant was centrifuged at $10,000 \times g$ for 30 min. to obtain crude mitochondrial pellet. The pellet was incubated with CHAPS of 1% at 37°C for 60 min. and centrifuged at $1,000 \times g$ for 15 min. The pellet was resuspended in 0.3 M sucrose and layered onto 1.2 M sucrose, and then centrifuged at $53,000 \times g$ for 2 h. Pellet was resuspended in KP buffer, pH 7.4, and kept at -70°C until used.

Measurement of total MAO activity

Total MAO activity was measured spectrophotometrically according to the method of Holt²⁷. The chromogenic solution consisted of 1 mM vanillic acid, 500 μM 4-aminoantipyrine, and 4 U. ml^{-1} pero-

xidase in 0.2 M potassium phosphate buffer, pH 7.6. Assay mixture contained 167 μl chromogenic solution, 667 μl substrate solution (500 μM p-tyramine) and 133 μl potassium phosphate buffer, pH 7.6. The mixture was preincubated at 37°C for 10 min. before the addition of enzyme. Reaction was initiated by adding the homogenate (100 μl), and increase in absorbance was monitored at 498 nm at 37°C for 60 min. Molar absorption coefficient of $4654 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used to calculate the initial velocity of the reaction. Results were expressed as $\text{nmol. h}^{-1} \cdot \text{mg}^{-1}$.

Selective measurement of MAO-A and MAO-B activities

Homogenates were incubated with the substrate p-tyramine (500 μM to measure MAO-A and 2.5 mM to measure MAO-B) following the inhibition of one of the MAO isoforms with selective inhibitors. Aqueous solutions of clorgyline or pargyline (50 μM), as selective MAO-A and -B inhibitor, were added to homogenates at the ratio of 1:100 (v/v), yielding the final inhibitor concentrations of 0.50 μM . Homogenates were incubated with inhibitors at 37°C for 60 min. prior to activity measurement. After incubation of homogenates with selective inhibitors, total MAO activity was determined by the method described above.

Determination of H_2O_2 production

H_2O_2 generation in lung homogenates was measured spectrofluorimetrically²⁸. Homogenates in 0.2 M KP buffer, pH 7.4, containing 1 mM homovanillic acid and 5 U. ml^{-1} horseradish peroxidase, were preincubated at 37°C for 10 min. Tyramine (10-80 μM) was added and H_2O_2 accumulation in the medium was measured after 30 min. The reaction was stopped by adding 0.1 N NaOH. Fluorescence was measured at $\lambda_{\text{ext}} = 323 \text{ nm}$ and $\lambda_{\text{em}} = 426 \text{ nm}$. Results were expressed as nmol. mg^{-1} .

Protein determination

Protein contents of the homogenates were determined according to the method of Bradford²⁹, in which bovine serum albumin was used as standard.

Statistical analysis

The results were expressed as the mean±SEM and analyzed by SPSS (version 9.0). Mann-Whitney U test and one-way analysis of variance (ANOVA) were used for comparison of the groups of the variables. Correlations between variables were assessed with Pearson's correlation coefficients (r), and $p < 0.05$ was considered as statistically significant.

RESULTS and DISCUSSION

MDA concentration was found to be significantly increased in the I/R group when compared with that of the control group ($p < 0.01$), and was significantly decreased in the IP group when compared with that of the I/R group ($p < 0.01$) (Table 1). This result was in good agreement with the previous reports demonstrating an increase of ROS production in I/R and protection of organs by IP against ROS-mediated I/R injury^{7-9,30,31}. It has been postulated

that although ischemia causes excess generation of ROS, which may contribute to direct cellular oxidant damage, the same source of ROS somehow triggers preconditioning and leads to an adaptation, including the enhancement of natural defense mechanisms^{31,32}.

GSH content and the GSH/GSSG ratio were found to be significantly decreased and GSSG content significantly increased in the I/R group ($p < 0.01$) (Table 1). GSH content and GSH/GSSG ratio were found to be significantly increased and GSSG content significantly decreased in the IP group ($p < 0.01$), suggesting that IP reduces the glutathione depletion in I/R and protects the lung against I/R injury. This finding was in good agreement with a previous report suggesting that IP causes a marked increase in GSH and decrease in GSSG levels¹¹. Strong negative correlations between MDA and GSH levels ($r = -0.56; -0.65; -0.67$ in control, I/R and IP groups, respectively, $p < 0.05$) and between MDA levels and GSH/GSSG ratio ($r = -0.60; -0.65; -0.70$ in control, I/R

Table 1. Lipid peroxidation, glutathione levels and MAO activities in lung tissues of the study groups*

Parameters	Control group	I/R group	IP group
MDA (nmol.mg ⁻¹)	41.23±3.56	83.29±6.01 ^a	42.33±3.05 ^b
GSH (µmol.mg ⁻¹)	64.23±5.12	30.58±6.15 ^a	61.16±4.76 ^b
GSSG (µmol.mg ⁻¹)	6.04±1.31	18.68±4.00 ^a	7.20±1.01 ^b
GSH/GSSG	8.97±2.02	2.06±0.93 ^a	8.11±1.03 ^b
CAT (nmol.mg ⁻¹)	68.56±5.30	45.22±5.30 ^a	59.70±6.32 ^{b,c}
Total MAO (nmol.h ⁻¹ .mg ⁻¹)	18.22±1.40	39.88±3.35 ^a	19.18±2.00 ^b
MAO-A (nmol.h ⁻¹ .mg ⁻¹)	9.23±0.84	28.13±1.80 ^a	10.03±1.25 ^b
MAO-B.(nmol.h ⁻¹ .mg ⁻¹)	7.01±0.64	13.02±1.43 ^a	8.06±0.73 ^b
H ₂ O ₂ (nmol. mg ⁻¹)	7.96±0.60	28.18±2.00 ^a	9.36±1.57 ^b

MDA: malondialdehyde; GSH: reduced glutathione; GSSG: oxidized glutathione; CAT: catalase;

MAO: monoamine oxidase; I/R: ischemia/reperfusion; IP: ischemic preconditioning

* Each group consisted of 6 rats. Values represent the mean±SEM.

^a $p < 0.01$ versus the control group

^b $p < 0.01$ versus the I/R group

^c $p < 0.05$ versus the control group

and IP groups, respectively, $p < 0.05$), and also the strong positive correlations between MDA and GSSG levels ($r = 0.59; 0.62; 0.69$ in control, I/R and IP groups, respectively, $p < 0.05$) supported our hypothesis above.

As seen in Table 1, total MAO, MAO-A and MAO-B activities were significantly elevated following 2 h of ischemia reperfusion in the rat isolated lung. In earlier reports, it was suggested that MAO activity and MAO-dependent H_2O_2 generation were strongly inhibited in renal and brain tissues, possibly due to accumulation of neurotransmitters such as serotonin, dopamine and noradrenaline during ischemia^{21,33}. Our results indicating the marked increase in tissue MAO activity (particularly in MAO-A activity) during the early reperfusion period in the rat lung were found to be in accordance with these previous reports. However, a detailed assay protocol and time course are needed to determine whether MAO is depressed or activated in ischemia protocol and in early and late phases of reperfusion. MAO activities were found to be significantly decreased and almost reached basal level in the IP group when compared with that of the I/R group ($p < 0.01$) (Table 1). A strong positive correlation was found between the tissue MAO-A and -B activities and MDA level in the I/R (for MAO-A and MDA $r = 0.73$; for MAO-B and MDA $r = 0.60$, $p < 0.01$) and IP (for MAO-A and MDA $r = 0.70$ and for MAO-B and MDA $r = 0.61$, $p < 0.01$) groups. This finding indicated that elevated MAO activity in ischemia reperfusion could cause excessive ROS production, which leads to lipid peroxidation in corresponding tissues. The data also showed that IP protected the tissues against oxidative damage by preventing the MAO activation. Previous studies suggesting that the start of lipid peroxidation of biological membranes by excessive hydroxyl radicals generated from the activation of MAO is sufficient to trigger a cascade of reactions leading to cell damage³⁴ supported our recent data.

MAO-induced H_2O_2 production in lung tissues of study groups were determined by a spectrofluorimetric technique. It was found that H_2O_2 release in lung tissues was significantly increased in I/R and

decreased in IP subgroups ($p < 0.01$) (Table 1). Incubation of homogenates with tyramine caused a concentration-dependent increase in peroxide accumulation in all study groups, whereas the plateau of H_2O_2 was obtained at tyramine concentrations from 60 to 80 μM in the I/R and 50 to 60 μM in the IP groups (Fig. 2). Tissue MAO-A and -B activities of lungs were positively correlated with H_2O_2 release in I/R (for MAO-A and H_2O_2 $r = 0.70$; for MAO-B and H_2O_2 $r = 0.59$, $p < 0.01$) and IP (for MAO-A and H_2O_2 $r = 0.72$ and for MAO-B and H_2O_2 $r = 0.61$, $p < 0.01$) groups, demonstrating that elevated MAO activity, which was possibly induced by increased neurogenic amines in damaged tissues, caused excess H_2O_2 release in I/R. Since excessive formation of free radicals in the early phase of reperfusion following ischemia has been documented in a number of organs^{1-4, 20,21} and since recent studies have shown that MAOs contribute to increase in H_2O_2 production²¹, it seems possible that MAO isoforms, particularly MAO-A, are responsible for the ROS-mediated tissue injury during I/R of the lung.

As tissue catalase is the major antioxidant which neutralizes ROS³⁵, and it is suggested that IP can activate antioxidant enzymes³⁶, tissue catalase activity was measured both in I/R and IP groups in order to determine the antioxidant response of the lung to excessive MAO-dependent H_2O_2 generation. It was found that catalase activity was significantly decreased in the I/R group when compared with that of the control group ($p < 0.01$), and increased in the IP

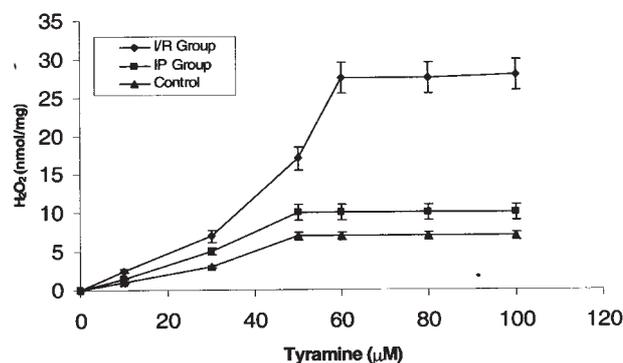


Figure 2. MAO-dependent tyramine degradation and H_2O_2 production in lung homogenates of study groups. Values represent the mean \pm SEM of six different experiments.

group when compared with that of the I/R group ($p < 0.01$). However, declined catalase activity of lung tissue in I/R could not reach its basal level with IP, suggesting that IP may not be completely successful in enhancing the antioxidant capacity of the lung against ROS-mediated I/R injury (Table 1). A weak negative correlation was found between tissue H_2O_2 generation and catalase activity in the I/R group, while no correlation was detected between these parameters in the IP group (data not shown). This result demonstrated that the increased catalase activity through H_2O_2 stimulation may not be operative in cytoprotection by IP against lethal H_2O_2 stress. We suggest that enhancement of antioxidant content by the IP process has only limited protective effect against H_2O_2 -mediated tissue injury and also that MAO inhibitors plus IP may be more effective for protection against I/R injury in the lung.

In summary, results of the present study demonstrated a significant ROS-mediated tissue injury in I/R and suggested that MAOs may be one of the potential sources of excessive H_2O_2 generation in the reperfusion period of I/R in the lung. IP was found to be an effective process to prevent excess ROS generation possibly caused by activated MAOs. If the MAO-mediated increase in H_2O_2 release is indeed involved in I/R damage of the lung, and IP is an effective procedure for preventing I/R injury, then pre-treatment with specific MAO inhibitors prior to or together with IP may have potential clinical relevance.

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