Effect of carbon dioxide pneumoperitoneum on the expression and activity of arginase in rats

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< ABSTRACT >

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Oxidative stress during carbon dioxide (CO_2) pneumoperitoneum (PP) is reported to be associated with decreased bioavailability of nitric oxide (NO). However, the changes in endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and arginase during different intraabdominal pressures (IAP) of CO_2 PP have not been elucidated.

Thirty male Sprague-Dawley rats were randomized into three groups. After induction of anesthesia, the abdominal cavity of the rats of group IAP-10 and IAP-20 were insufflated with CO_2 at pressures of 10 mmHg and 20 mmHg, respectively for 2 hours, while rats of group IAP-0 were not insufflated. After desufflation, plasma nitrite levels were measured while protein expression levels and activity of eNOS, iNOS, arginase I (Arg I) and arginase II (Arg II) were analyzed with aorta tissue samples. Tissue malondialdehyde (MDA) levels

were also measured.

Plasma nitrite levels and the expression of eNOS were significantly suppressed in groups IAP-10 and IAP-20 compared to group IAP-0. iNOS expression was found to be comparable between the three groups. Arg I and Arg II expression was significantly increased in groups IAP-10 and IAP-20 compared to group IAP-0. eNOS activity was significantly reduced in groups IAP-10 and IAP-20 compared to group IAP-0. Arginase activity was significantly increased in group IAP-20 compared to groups IAP-0 and IAP-10. MDA levels were also significantly increased in group IAP-20 compared to groups IAP-0 and IAP-10.

eNOS expression and activity decreases during CO_2 PP, which leads to a decrease in NO bioavailability and increased oxidative stress. Arginase expression and activity is increased during CO_2 PP, which seems to act reciprocally with the NO system.

Key words: arginase, nitric oxide synthase, nitric oxide, pneumoperitoneum

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I. INTRODUCTION

The benefits of laparoscopic surgery such as reduced postoperative pain, faster recovery and shorter hospital stay, and improvement in cosmetic outcome are now uncontroversial.[1-3] Moreover, the rapid advancement of surgical techniques and improved equipment has extended its application to a wide variety of diseases and patients that were once considered as contraindications to laparoscopic surgery.[4-7] However, laparoscopic surgery requires carbon dioxide (CO_2) insufflation into the peritoneal cavity in order to secure a surgical work space, with intra-abdominal pressures (IAP) usually maintained at 10~15 mmHg. Although well-tolerated by most patients for even an extended period of time, this state of CO_2 pneumoperitoneum (PP) is clearly non-physiological and is well known to trigger a number of stress responses in the human body such as myocardial suppression and decreased cardiac output,[8] sympathetic nervous system (SNS) activation,[9-11] decreased splanchnic blood flow and possible organ ischemia due to oxidative stress.[12-16]

The role of decreased nitric oxide (NO) availability as a mechanism of

splanchnic ischemia during PP has recently received much attention. Nitric oxide synthase (NOS) is the rate-limiting enzyme involved in the conversion of L-arginine to NO. Among the three NOS isoforms neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), eNOS is the predominant isoform in the vasculature and is responsible for most of the NO produced in this tissue.[17] NO plays a critical role in central autonomic control.[18] It also functions as a vasodilator and has antithrombotic, anti-inflammatory, antiproliferative and antioxidant properties.[19] The alterations in splanchnic blood flow and increased oxidative stress due to PP was proven to be associated with the decreased bioavailability of NO in several studies. Inclusion of ethyl nitrite (ENO), a donor substance of NO in the insufflation gas was reported to be effective in reducing hepatic and renal ischemia caused by pneumoperitoneum,[20,21] Pretreatment with nitroglycerine ameliorated the adverse renal effects after PP,[13,22] while pretreatment with NG-nitro-L-arginine methyl ester (L-NAME), which is an inhibitor of NOS, was shown to aggravate renal ischemia.[13,23] On the other hand, iNOS is up-regulated in macrophages and virtually any cell or tissue during various types of inflammatory diseases.[24] NO derived from iNOS mediates various symptoms of inflammation, and has been reported to be significantly more increased after laparoscopy compared to laparotomy.[25]

While the above-mentioned studies focus on the activity of eNOS, iNOS and NO production itself, decreased NO synthesis may also be affected by the

enzyme arginase. Because arginase hydrolyses L-arginine to ornithine and urea as part of the urea cycle, arginase is thought to inhibit NO synthesis by competing with NOS for L-arginine.[26,27] The inhibition of arginase has been shown to stimulate NO synthesis, while the overexpression of arginase suppresses NO generation in endothelial cells.[26] Arginase exists in two isoforms; arginase I (Arg I) and arginase II (Arg II). While these isoforms seem to be regulated independently and have different roles in various pathophysiological conditions, both have been reported to be associated with endothelial dysfunction and decreased NO bioavailability likely by regulating substrate availability.[28-30] In this context, the decreased bioavailability of NO during PP can be hypothesized to be related to the relative up-regulation of arginase. Also, the increase in arginase activity during PP and the following depletion of substrate may cause eNOS to produce reactive oxygen species, thus worsening oxidative stress in a vicious cycle.

Although the relationship between decreased NO and splanchnic ischemia has been studied and supported by several recent studies, the changes in specific NOS isoforms have not been elucidated. Furthermore, the role of arginase and its reciprocal actions between eNOS during PP is a new field of interest that may serve as a novel target for reducing oxidative stress due to laparoscopic surgery. This study was done to investigate the changes in eNOS, iNOS and arginase, NO bioavailability and degree of oxidative stress during CO_2 PP of different IAPs in rats.

II. MATERIALS AND METHODS

1. Animals and experimental design

All procedures of this study were approved by the Yonsei University Institutional Animal Care and Use Committee. Experiments were performed on 30 male Sprague-Dawley rats that were 3 ~ 4 months old, weighing 250 ~ 320 g. The rats were allowed to adjust to the controlled laboratory environment of 22 ~ 24 ℃ for at least 7 days with free access to standard laboratory chow and tap water prior to experiments. After the rats were anesthetized with 2 % of sevoflurane inhalation in the supine position, they were safely secured on a thermoregulated pad (37 ℃) and mechanically ventilated with a volume-driven small-animal ventilator (model 665A, Harvard Apparatus, Holliston, MA) after tracheotomy. Tidal volume was set between 0.5 and 1 mL/100g of body weight and the respiratory rate was adjusted at 55 ~ 60 breaths/min to maintain end-tidal CO_2 at 30 ~ 35 mmHg. Anesthesia was maintained with 1.5 ~ 2.0 % sevoflurane with 50 % oxygen in air throughout the study. The abdominal cavities of the rats were then punctured with a Veress needle that was connected to an electronic laparoflator for CO_2 insufflation and the rats were randomized to three groups of 10 animals each. The IAPs of the rats in group IAP-10 and IAP-20 were maintained at 10 mmHg and 20 mmHg, respectively for 2 hours, while the rats of group IAP-0 were not insufflated with CO_2. Blood samples were collected at the end of the procedure after the release of PP. The rats were

then euthanized by exsanguination and the aorta was harvested and dissected from connective tissue in Kreb solution.

2. Nitric oxide analysis

Nitrite (NO^{2-}) is a stable metabolite of NO and the most suitable and practical method to assess NO synthesis *in vivo*. Blood was collected in tubes using citrate, EDTA as an anticoagulant and was centrifuged for 15 minutes at 1000 g of collection. To measure nitrite, plasma was incubated with 100 μL of Griess reagent (1 % sulfanilamide in 0.1 mol/L HCl and 0.1 % N-(1-naphthyl) ethylene diamine dihydrochloride, Sigma-Aldrich, MO, USA) at room temperature for 10 minutes. Then the absorbance was measured at 540 nm using a microplate reader. The nitrite content was calculated based on a standard curve constructed with $NaNO_2$.

3. Western blot analysis

Abdominal aorta tissue was homogenized in ice-cold Proprep (Intron Biotechnology, Korea). Aorta extracts were loaded on 8 ~ 10 % SDS-polyacrylamide gel. The proteins were transferred on polyvinyl-difluoride membranes (0.2 μm: Immun-Blot, Bio-Rad, CA, USA). Membranes were blocked using 5% (wt/vol) nonfat milk in Tris-buffered saline with 0.1 % Tween-20. The blots were incubated overnight at 4 ℃ with primary antibodies, eNOS (Santa Cruz Biotechnology, CA, USA), iNOS (Santa Cruz Biotechnology,

CA, USA), Arg I (R&D Systems, MN, USA), and Arg II (Santa Cruz Biotechnology, CA, USA) at 1:1,000 dilution. After incubation, the membranes were incubated with anti-rabbit, anti- sheep or anti-mouse secondary antibodies at 1:5,000 dilution. Protein expression was normalized to β-actin (1:1,000 dilution). The bands were detected using a chemiluminescence assay (ECL Plus, Amersham Biosciences, CA, USA).

4. Endothelial nitric oxide synthase activity

Activity of eNOS was assayed by a rat eNOS ELISA kit (Wuhan EIAab Science Co., Ltd, Wuhan, China). In brief, the supernatants of aorta tissue were homogenized in ice-cold Proprep (Intron Biotechnology, Korea), and then incubated in a 96 well plate containing the co-factors and substrate L-arginine, for 1 hour at 37 ℃. After the incubation period, the reaction was quenched by the addition of the stop buffer. The concentration of nitrites and nitrates in the reaction mixture was determined by the colorimetric method (530 nm) to evaluate eNOS activity.

5. Arginase activity

Activity of intracellular arginase was determined from aorta digest cell lysates by the QuantiChrom™ Arginase Assay Kit (BioAssay Systems, CA, USA), which measures the conversion of arginine to urea by arginase. After total protein quantification, the sample was incubated with arginine buffer at 37 ℃

for 2 hours. Optical density was read using a 430 nm filter.

6. Malondialdehyde analysis

Malondialdehyde (MDA) is a stable product of lipid peroxidation, which is regarded as an index of oxygen free radical production. Aorta tissue MDA was analyzed using an OxiSelect™ MDA Adduct ELISA kit (Cell Biolabs, Inc. CA, USA). Absorbance was read by a microplate reader using 450 nm as the primary wave length after adding Stop Solution to stop the enzyme reaction.

7. Statistical analysis

Data are described as mean ± SD or numbers of subjects. Nonparametric data such as proportion of muscular pulmonary arteries were analyzed with the chi-square test. ANOVA with Tukey's multiple comparison was performed to compare parametric variables among the three groups. *P* values less than 0.05 were considered statistically significant.

III. RESULTS

1. Nitric oxide

Plasma nitrite levels were significantly reduced in rats of group IAP-10 and IAP-20 compared to group IAP-0 (8.4 ± 2.1 μmol/mL and 8.7 ± 2.9 μmol/mL vs. 14.0 ± 3.2 μmol/mL, respectively, $P < 0.01$ in both). Plasma nitrite was highest in group IAP-0 among all groups while there was no difference between groups IAP-10 and IAP-20 (Fig 1).

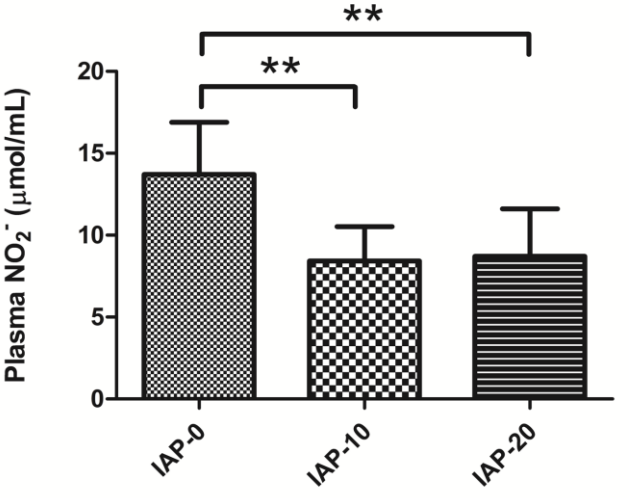

Figure 1. Effect of CO_2 pneumoperitoneum at different IAPs on plasma nitrite. Plasma nitrite levels were significantly reduced in groups IAP-10 and IAP-20 compared to group IAP-0. There was no difference between groups IAP-10 and IAP-20. ** $P < 0.01$

2. Endothelial nitric oxide synthase, inducible nitric oxide synthase, arginase I and arginase II expression

The expression of eNOS was significantly suppressed in groups IAP-10 and IAP-20 compared to group IAP-0 (1.3 ± 0.10 and 0.90 ± 0.26 vs. 1.8 ± 0.21, respectively, $P < 0.05$ and $P < 0.01$, respectively), while there was no difference between groups IAP-10 and IAP-20 (Fig 2B). The expression of iNOS was found to be comparable between the three groups (3.1 ± 0.88, 2.2 ± 1.1 and 2.0 ± 0.93 in groups IAP-0, IAP-10 and IAP-20, respectively) (Fig 2C). There was no significant difference in Arg I expression between the three groups (2.0 ± 0.80, 1.3 ± 0.50 and 1.2 ± 0.23 in groups IAP-0, IAP-10 and IAP-20, respectively) (Fig 2D). Arg II expression was significantly increased in group IAP-20 compared to group IAP-0 (5.0 ± 1.5 vs. 1.3 ± 0.61, $P < 0.05$), but there was no difference between groups IAP-0 and IAP-10 or IAP10 and IAP-20 (Fig 2E).

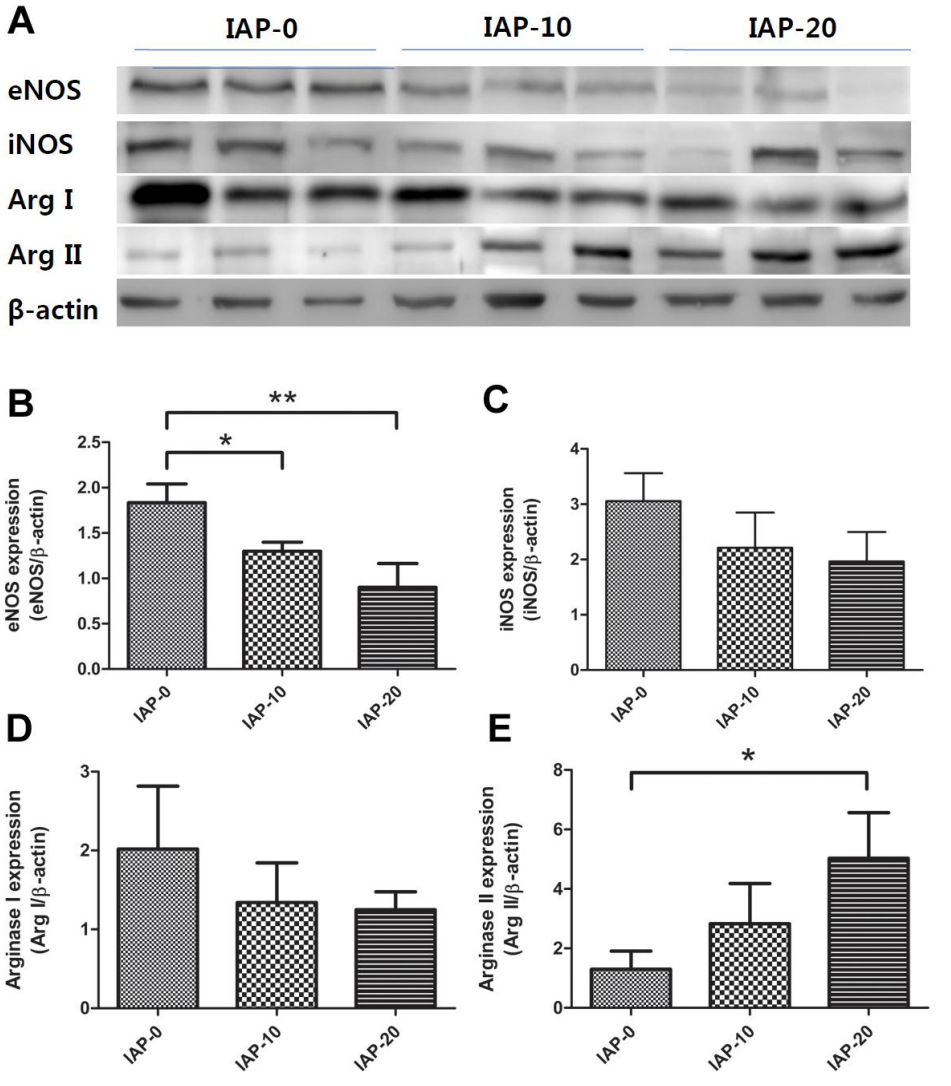

Figure 2. Effect of CO_2 pneumoperitoneum at different IAPs on eNOS, iNOS, Arg I and Arg II protein expression. (A) Western blot for eNOS, iNOS, Arg I, Arg II and β-actin (internal reference) in rat aorta tissue after different intraabdominal pressures. (B) The expression of eNOS was significantly suppressed in groups IAP-10 and IAP-20 compared to group IAP-0, while there

was no difference between groups IAP-10 and IAP-20. (C) There was no significant difference in iNOS expression between the three groups. (D) There was no significant difference in Arg I expression between the three groups. (E) Arg II expression was significantly increased in group IAP-20 compared to group IAP-0. * $P < 0.05$, ** $P < 0.01$

3. Endothelial nitric oxide synthase and arginase activity

The activity of eNOS was significantly reduced in groups IAP-10 and IAP-20 compared to group IAP-0 (4.4 ± 0.77 U/L and 4.7 ± 0.39 U/L vs. 5.6 ± 1.1 U/L, respectively, $P < 0.01$ and $P < 0.05$, respectively), with no difference between groups IAP-10 and IAP-20 (Fig 3A). Arginase activity was significantly increased in group IAP-20 compared to groups IAP-0 and IAP-10 (0.54 ± 0.67 U/L vs. 0.09 ± 0.05 U/L and 0.09 ± 0.04 U/L, respectively, $P < 0.05$ in both). However, there was no significant difference in arginase activity between groups IAP-0 and IAP-10 (Fig 3B).

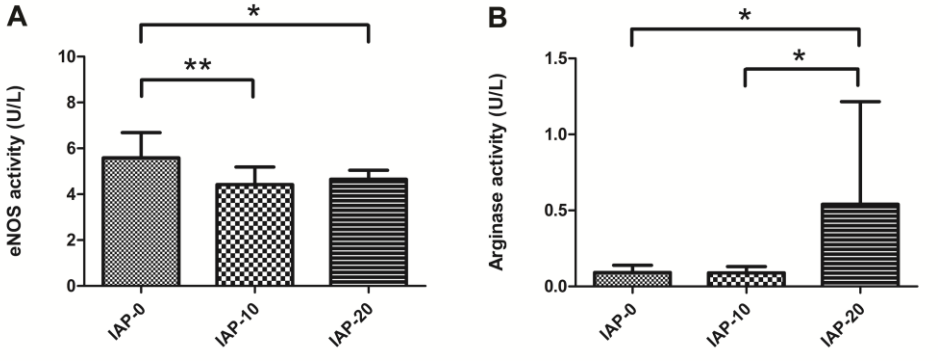

Figure 3. Effect of CO_2 pneumoperitoneum at different IAPs on eNOS and arginase activity. (A) The activity of eNOS was significantly reduced in groups IAP-10 and IAP-20 compared to group IAP-0, with no difference between groups IAP-10 and IAP-20. (B) Arginase activity was significantly increased in group IAP-20 compared to groups IAP-0 and IAP-10. No difference was seen between groups IAP-0 and IAP-10. * $P < 0.05$, ** $P < 0.01$

4. Malondialdehyde levels

MDA levels were significantly increased in group IAP-20 compared to groups IAP-0 and IAP-10 (1.4 ± 0.17 pmol/mg vs. 1.1 ± 0.09 pmol/mg and 1.2 ± 0.08 pmol/mg, respectively, $P < 0.001$ and $P < 0.01$, respectively). However, there was no difference in MDA levels between groups IAP-0 and IAP-10 (Fig 4).

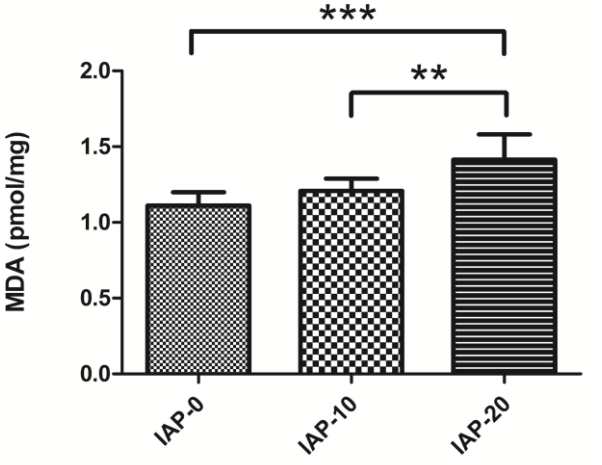

Figure 4. Effect of CO_2 pneumoperitoneum at different IAPs on tissue MDA

levels. MDA levels were significantly increased in group IAP-20 compared to groups IAP-0 and IAP-10. There was no difference between groups IAP-0 and IAP-10. ** $P < 0.01$, *** $P < 0.001$

IV. DISCUSSION

The results of this study support the well-known decrease in NO bioavailability and increased oxidative stress during CO_2 PP, and shows that decreased expression and activity of eNOS is a main cause of this phenomenon. Moreover, our findings provide novel information regarding the increased expression and activity of arginase as an underlying mechanism of decreased NO bioavailability during laparoscopic surgery.

Several recent studies suggested the involvement of the NO system in the changes in renal function after CO_2 PP. Abassi et al.[13] demonstrated that the blockade of NOS with L-NAME aggravated PP-induced renal hypoperfusion and oliguria while pretreatment with nitroglycerine (NTG) attenuated this effect. Bishara et al.[22] reported that rats with decompensated congestive heart failure were more susceptible to the adverse renal effects of PP when pretreated with L-NAME.[23] They also suggested that NOS inhibition with L-NAME aggravates adverse renal effects of high but not low or moderate IAPs.[31] While all of these aforementioned studies concluded that NO and NOS may be strongly involved with the adverse effects of PP by evaluating markers of renal function as surrogates, actual tissue or plasma levels of NO were not evaluated. The results of our study support the results of the previous studies by showing the actual decrease in nitrite levels as well as the expression and activity of eNOS in rats that were insufflated with CO_2 (Fig 1, 2B, 3A). Interestingly, while the nitrite levels were both significantly decreased in groups IAP-10 and IAP-20

compared to group IAP-0, there was no difference between different the two insufflated groups. A compensatory increase in NO secretion at higher insufflations pressures in response to worsening hypoperfusion has been suggested in a recent study.[32] This may be a possible explanation for the similar plasma nitrite levels between groups IAP-10 and IAP-20 of the present study.

The involvement of iNOS during laparoscopic surgery is not well known, and the existing evidence show controversial results. While Hajri et al.[33] reported that 2 hours of CO_2 PP lead to the depression of iNOS mRNA production in rats, Romeo et al.[25] found increased iNOS activity and overexpression of mRNA for iNOS following 1 hour of CO_2 PP. Romeo et al. suggested a time-dependent effect, due to which the 2 hour CO_2 PP in the former study may have exhausted macrophage function and therefore iNOS activity. On the other hand, the production if NO during different stages of asthma has been explained by the upregulation of iNOS in the latter phase of asthmatic reaction.[34] The trends of iNOS expression and activity in different disease states are not clear, and further studies done at various lengths of CO_2 PP are needed to uncover the role of iNOS in oxidative stress caused by laparoscopic surgery.

As expected, the results of this study showed a significant increase in arginase activity at a high insufflation pressure of 20 mmHg (Fig 3). Arginase has received widespread interest due to its involvement in a wide range of physiological and pathophysiological conditions such as vascular disease, pulmonary disease, infectious disease and cancer.[35] The functional relevance of

arginase and the availability of L-arginine was even more emphasized due to the fact that NOS and arginase shares the same substrate.[26] The competition for L-arginine supply is intensified under acute and chronic stress conditions, contributing to the shortage of NO bioavailability.[36] While oxidative stress, tissue injury and reduction in kidney function during CO_2 PP has been suggested to be closely related to the NO system in recent studies,[12,22,31] the role of arginase during PP has not yet been elucidated. Arginase exists in two isoforms in mammals as Arg I and Arg II and these isoforms seem to be expressed at different sites to different degrees under various pathophysiological conditions. Although there is evidence suggesting that the predominant isoform detected in human endothelium is Arg II,[35] both types are reported to be involved in endothelial dysfunction and the downregulation of NO synthesis to some extent.[28-30] Interestingly, expression of Arg I was not significantly different between the three groups. However, Arg II showed a consistent trend of higher expression levels with increasing IAPs. Arg II expression was higher in group IAP-10 compared to group IAP-0, and this increase became clearly significant in group IAP-20. Moreover, a significant increase in arginase activity was observed in group IAP-20 compared to the other two groups. This may implicate a stronger association of Arg II than Arg I with impaired endothelial NO production during CO_2 PP, suggesting that it may be a more effective target for the attenuation of NO synthesis during laparoscopic surgery.

Oxidative stress and its determinants during CO_2 PP have been reported in

many animal models as well as human subjects.[12] The impact of insufflation pressure on the degree of oxidative stress is well known, and thus using the lowest possible inflation pressure is recommended.[12] Higher IAPs have been shown to contribute to more severe intestinal mucosal injury by causing ischemia during pneumoperitoneum, but also after deflation by triggering a burst of ROS production.[37] The results of this study agree with the previous findings by showing higher levels of tissue MDA after higher levels of inflation pressures (Fig 4). It is noteworthy that splanchnic ischemia due to oxidative stress has even been reported in previously healthy patients,[12] and that is may possibly extend to extraabdominal organs as well.[38] The present study shows that arginase upregulation is clearly among the many mechanisms of oxidative stress, and this finding offers a potential target for treatment and prevention of ischemia-reperfusion injury during laparoscopic surgery. Recently, arginase inhibitors such as 2(S)-amino-6-boronohexanoic acid (ABH) or N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) have been found to be effective for the treatment of various cardiovascular diseases[39] and even protection against hepatic[40] and cardiac[41] ischemia-reperfusion injury in experimental and clinical studies with positive results. Further studies on the application of arginase inhibitors in laparoscopic surgery for protection against oxidative stress due to ischemia-reperfusion may yield positive results for safer surgery in high-risk patients.

This study has several limitations. Firstly, while the results of this study

clearly show a reciprocal action between eNOS and arginase during CO_2 PP, uncovering the mechanism underlying the exact interaction between the two enzymes was beyond the scope of this study. The exact role of arginase and its metabolism in the human body itself is not yet completely understood and further research is needed to elucidate the significance of this enzyme in many pathophysiological states including CO_2 PP. Secondly, many other factors that may affect the extent of oxidative stress such as different durations of CO_2 PP, nature of the gas used and position of the patient were not studied. Due to the experimental design of this study, we were not able to evaluate the changes in the activity and expression of enzymes or levels of oxidative stress in a sequential manner, but only at a certain time point. A study with subdivided groups is needed to provide more information regarding the impact of various factors that may lead to different amounts of oxidative stress. Thirdly, this study was conducted in healthy rats and therefore the results may not accurately represent the patterns of eNOS or arginase expression in humans or diseased conditions. While arginase is constitutively expressed in endothelial cells, the expression of specific isoforms is known to differ among mammalian species.[35] Lastly, the expression of iNOS was studied with abdominal aorta tissue of the rats in this study. The main cellular source for iNOS during the inflammatory process is thought to be macrophages,[24] and therefore studying iNOS expression and activity in peritoneal macrophages may have offered more insight into its involvement during CO_2 PP.

V. CONCLUSION

In conclusion, our study provides evidence that eNOS expression and activity decreases during CO_2 PP, which leads to a decrease in NO bioavailability and increased oxidative stress. Moreover, our findings demonstrate that arginase expression and activity is increased during CO_2 PP, which seems to act reciprocally with the NO system. These results offer a new potential target for attenuating the adverse outcomes of laparoscopic surgery. Studies conducted in subjects of diseased states such as congestive heart failure or vascular diseases may help to anticipate the possible effectiveness of arginase inhibition during laparoscopic surgery in patients at high risk of oxidative stress and its side effects.

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< ABSTRACT (IN KOREAN) >

백서에서 이산화탄소 기복증이 arginase의 발현과 활성에 미치는 영향

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신서경

이산화탄소 기복 중 발생하는 산화스트레스는 산화질소 (NO)의 감소와 연관이 있는 것으로 알려져 있다. 그러나 각기 다른 복압의 이산화탄소 기복 중에 발생하는 내피세포 산화질소합성효소 (endothelia nitric oxide synthase, eNOS), 유도형 산화질소합성효소 (inducible nitric oxide synthase, iNOS) 및 arginase의 변화는 아직 밝혀진 바가 없는 상태이다.

3 ~ 4 월령의 백서 30 마리를 IAP-0, IAP-10 및 IAP-20 군의 세 군 중 하나로 배정하였다. 전신 마취 유도 후, IAP-10 및 IAP-20 군의 백서들은 각각 10 mmHg 또는 20mmHg의 복압으로 2 시간 동안 이산화탄소 기복을 유지하였으며, IAP-0 군은 기복을 유도하지 않았다. 2 시간의 기복이 종료된 후 혈장 NO 농도를 측정하였으며 대동맥 조직을 채취하여 eNOS, iNOS, arginase I (Arg I) 및 arginase II (Arg II)의 단백질 발현 정도와 활성도를 비교 분석하였다. 조직 malondialdehyde (MDA) 또한 측정하였다.

IAP-0 군과 비교하여 IAP-10 및 IAP-20 군에서 혈장 NO 및 eNOS의 발현이 유의하게 감소된 상태였으며, iNOS의 발현은 세 군간 차이가 없었다. Arg I 및 Arg II의 발현은 IAP-10 및 IAP-20 군에서

IAP-0 군에 비해 유의하게 증가되어 있었다. IAP-10 및 IAP-20 군에서 IAP-0 군에 비해 eNOS의 활성도가 유의하게 감소되어 있었으며, arginase의 활성도는 IAP-20 군에서 IAP-0 및 IAP-10 군에 비해 유의하게 증가되어 있었다. 조직 MDA는 IAP-20 군에서 IAP-0 및 IAP-10 군에 비해 유의하게 증가되어 있었다.

이산화탄소 기복은 eNOS의 발현 및 활성도를 감소시키며, 이는 NO의 감소와 산화스트레스의 증가를 초래한다. Arginase의 발현 및 활성도는 이산화탄소 기복 중에 증가하며 이는 NO 시스템과 상반되게 작용하는 것으로 생각된다.

핵심되는 말: arginase, 산화질소합성효소, 산화질소, 기복