Remifentanil Preconditioning Reduces Hepatic Ischemia–Reperfusion Injury in Rats *via* Inducible Nitric Oxide Synthase Expression

Li-Qun Yang, M.D.,* Kun-Ming Tao, M.B.B.S.,† Yan-Tao Liu, M.B.B.S.,†
Chi-Wai Cheung, M.B.B.S., F.H.K.A.M.,‡
Michael G. Irwin, M.B.Ch.B., M.D., F.R.C.A., F.A.N.Z.C.A., F.H.K.A.M.,§
Gordon T. C. Wong, M.B.B.S., F.A.N.Z.C.A.,‡ Hao Lv, M.B.B.S.,† Jian-Gang Song, M.D.,†
Fei-Xiang Wu, M.D.,† Wei-Feng Yu, M.D., Ph.D.‖

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ABSTRACT

Background: Opioid preconditioning against ischemia reperfusion injury has been well studied in myocardial and neuronal tissues. The objective of this study was to determine whether remifentanil could attenuate hepatic injury and to investigate the mechanisms.

Methods: A rat model of hepatic ischemia reperfusion injury and a hepatocyte hypoxia reoxygenation (HR) injury model were used, respectively, in two series of experiments. Remifentanil was administered before ischemia or hypoxia and the experiments were repeated with previous administration of naloxone, L-arginine and *N*-ω-nitro-L-arginine methyl ester, a nonselective opioid receptor antagonist, a nitric oxide donor, and nitric oxide synthase (NOS) inhibitor, respectively. Serum aminotransferase, cytokines, and hepatic lipid peroxidation were measured. Histopathology examination and apoptotic cell detection were assessed. For the *in vitro* study, cell viability, intracellular nitric oxide, apoptosis, and NOS expression were evaluated.

Results: Remifentanil and L-arginine pretreatment reduced concentrations of serum aminotransferases and cytokines, decreased the concentrations of hepatic malondialdehyde and myeloperoxidase activity, and increased superoxide dismutase, nitric oxide, and inducible NOS expression *in vivo*. Decreased histologic damage and apoptosis were also seen in these two groups. These changes were prevented by previous administration of *N*-ω-nitro-L-arginine methyl ester but not naloxone. There was an increase in inducible NOS protein expression but not endogenous NOS in remifentanil and L-arginine pretreated groups compared with control, naloxone, and *N*-ω-nitro-L-arginine methyl ester groups.

Conclusion: Pretreatment with remifentanil can attenuate liver injury both *in vivo* and *in vitro*. Inducible NOS but not opioid receptors partly mediate this effect by exhausting reactive oxygen species and attenuating the inflammatory response.

What We Already Know about This Topic

- Opioids have been shown to induce preconditioning in myocardial and neuronal tissues.
- Hepatic ischemic injury is frequently encountered in clinical settings, including hepatic resection, liver transplantation, and shock.

What This Article Tells Us That Is New

- Remifentanil can attenuate liver ischemic injury both *in vivo* and *in vitro*. Inducible nitric oxide synthase but not opioid receptor partly mediate this preconditioning effect.

HEPATIC ischemia is encountered in a number of clinical settings, such as hepatic resection, liver transplantation, and hemorrhagic shock.[1] Subsequent reperfusion can then exacerbate such injury.[2] The mechanisms of hepatic ischemia–reperfusion (I/R) injury have not been completely clarified, although there is evidence that Kupffer cell activation initiates the injury. This is followed by the generation of reactive oxygen species and the release of endogenous inflammatory mediators that trigger various molecular cascades and

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* Associate Professor, † Assistant Professor, § Professor and Head, Department of Anesthesiology, Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University, Shanghai, China. ‡ Assistant Professor, ‖ Professor and Director, Department of Anesthesiology, The University of Hong Kong, Hong Kong.

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Address correspondence to Dr. Yu: Department of Anesthesiology, Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University, 225# Changhai Road, Shanghai 200438, China. ywf808@sohu.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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activation of downstream mediators and cytokines that culminate in an imbalance between proinflammatory and anti-inflammatory responses.[3,4]

Administration of opioids such as morphine, fentanyl, or remifentanil can initiate an opioid receptor (OR)-mediated preconditioning effect in myocardium and nerve cells.[5–7] However, whether this beneficial effect occurs in the liver is not known. Remifentanil, as an ultra-short-acting phenylpiperidine opioid analgesic agent, is particularly popular because of its titratable pharmacokinetic profile that allows high intraoperative dosing without any delay in recovery. Remifentanil can suppress the inflammatory response and inhibit inducible nitric oxide synthase (iNOS) expression in a septic mouse model.[8] It has also been reported that remifentanil reduced the release of biomarkers of cardiac injury in patients undergoing cardiopulmonary bypass.[9] So it is possible that remifentanil could produce hepatoprotection through modulation of the inflammatory response and nitric oxide synthase (NOS) system.

The objective of our study was to determine whether remifentanil confers hepatic protection against I/R injury, and if so, whether NOS mediates this effect. To investigate the role of ORs in this process, we repeated the experiments in the presence of the nonselective OR antagonist naloxone. Because hepatic nonparenchymal cells can also express opioid receptors,[10] we used a hypoxia reoxygenation (HR) injury model to elucidate the mechanism in isolated rat hepatocytes.

Materials and Methods

Animals

This study was conducted in accordance with our institutional guidelines on the use of live animals for research, and the experimental protocol was approved by the Animal Care and Use Committee of the second Military Medical University, Shanghai, China. Male Sprague-Dawley rats weighing 250–300 g were housed in groups of three to four in a cage where temperature was controlled at approximately 22°C in an alternating 12-h light/12-h dark cycle. Animals were allowed free access to food and water until the night before anesthesia.

Hepatic I/R Injury In Vivo

Surgical Preparation. Anesthesia was induced by intraperitoneal injection of pentobarbitone (50 mg · kg^{-1} body weight, Dainabot, Osaka, Japan) and maintained by repeat doses of 25 mg · kg^{-1} if necessary, based on animal movement. All the surgical procedures were performed under sterile conditions. After tracheotomy and tracheal intubation, mechanical ventilation was provided with a Harvard Apparatus Rodent Respirator (Harvard Apparatus, Boston, MA), with room air at 60–70 breaths/min. Body temperature was maintained using a heating pad placed under the animal to keep the rectal temperature at 36 ± 1°C. The caudal vein was cannulated to infuse 0.9% saline or drugs. I/R was produced by temporarily occluding the blood supply to the left lateral and median lobes of the liver, as previously described.[4] Laparotomy was carried out through a midline incision, and the ligamentous attachments from the liver to the diaphragm were severed and the liver exposed. Ischemia of the median and left lateral lobes of the liver was produced by clamping the corresponding vascular pedicle containing the portal vein and branches of the hepatic artery using an atraumatic microvascular clamp for 45 min. Other hepatic lobes were not handled during the procedure. This method produces ischemia to the left and median lobes of the liver, and it leaves the blood supply to the right and caudate lobes uninterrupted. At the end of the ischemia period, the vascular clamp was removed, and the liver was reperfused for 2 h. After reperfusion, the vena cava was opened and 3–5 ml of blood was collected in sterile syringes without anticoagulant and centrifuged to separate the serum. The serum samples were stored at −20°C for later batch analysis of hepatic function and cytokine assay. The liver was then perfused with cold saline through the portal vein. Ischemic left hepatic tissue samples were collected and the specimens were fixed in 10% formalin and embedded in paraffin for histologic studies, immediately frozen in isopentane and liquid nitrogen, then stored at −80°C for later analysis. The animals were then given an additional dose of pentobarbitone (50 mg) before being killed by exsanguination.

Study Groups and Experimental Protocol *In Vivo*. The study consisted of three series of experiments as shown in figure 1A. The first series was to determine whether the administration of remifentanil (GlaxoSmithKline Limited, Philadelphia, PA) limits liver I/R injury. Rats were randomly assigned to receive one of five treatments (n = 10):

1. Sham operation group (n = 10), laparotomy and dissection of the portal vein but not clamping; tissue and blood samples collected after 3 h.
2. I/R group (n = 10), laparotomy and dissection of the portal vein. Ischemia was induced in the median and left lateral hepatic lobes for 45 min, followed by a 2-h period of reperfusion, whereas 1 ml 0.9% saline was infused through the caudal vein before 30 min of occlusion.
3. Remifentanil preconditioning (RPC) group (n = 30), laparotomy and dissection of the portal vein. Remifentanil preconditioning was divided into 3 subgroups of 10: remifentanil 1 μg · kg^{-1} bolus followed immediately by a 15-min infusion of 0.2, 2, and 20 μg · kg^{-1} · min^{-1} (RPC1, RPC2, and RPC3 groups, respectively), finishing 10 min before the I/R procedure. All of these animals underwent the same I/R procedure.

Subsequently, to evaluate the involvement of NOS in remifentanil preconditioning, L-arginine, a nitric oxide donor (Sigma Chemical Company, St. Louis, MO) and *N*-ω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of

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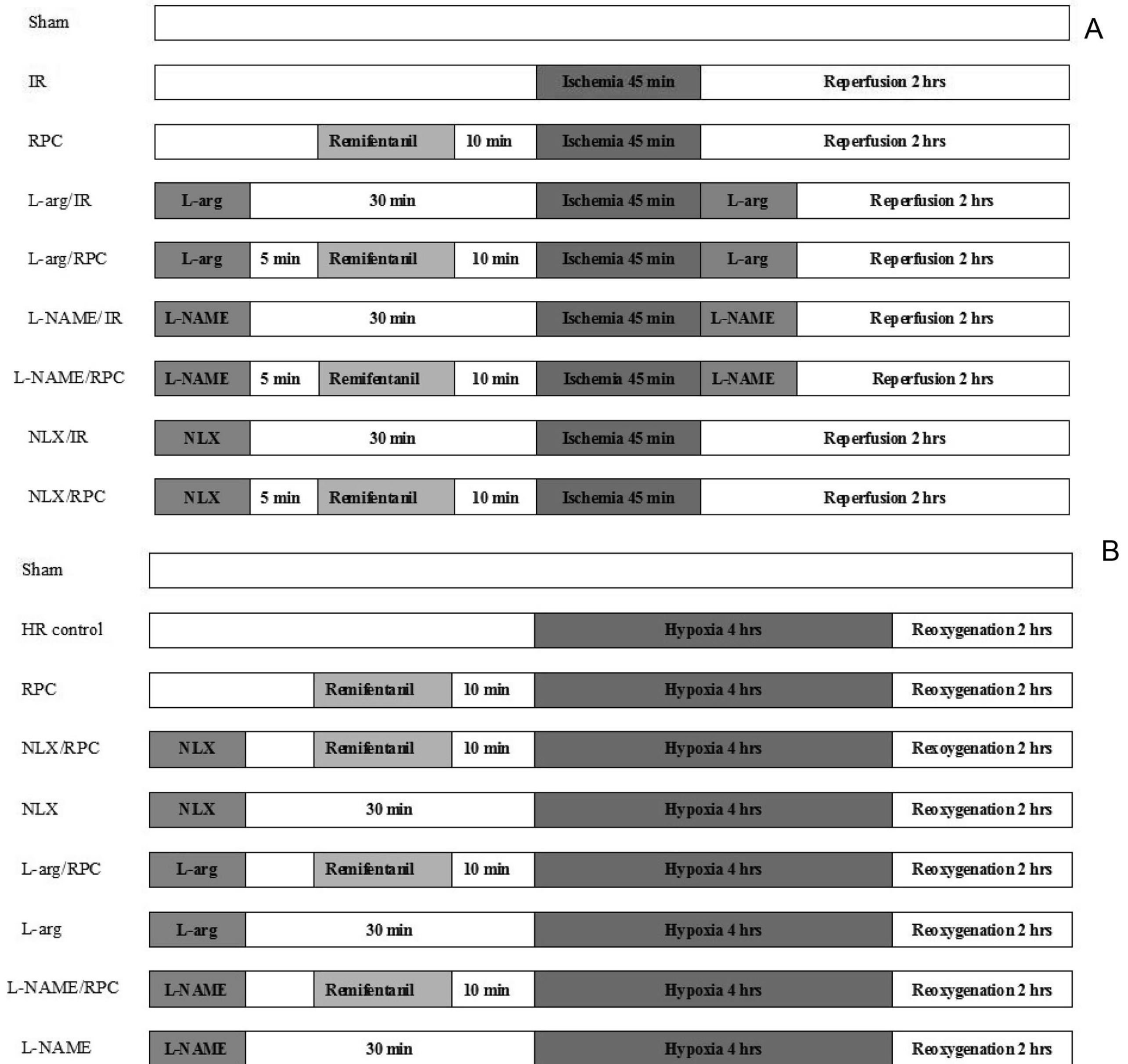

Fig. 1. The experiment protocols. (*A*) *In vivo* experiments are shown. (*B*) *In vitro* experiments are shown. HR = hypoxia and reoxygenation; IR = ischemia-reperfusion; L-arg = L-arginine; L-NAME = *N*-ω-nitro-L-arginine methyl ester; NLX = naloxone; R = reperfusion; RPC = remifentanil preconditioning.

NOS (Sigma Chemical Company). The rats were randomly assigned to one of four groups (n = 10) as follows:

1. L-Arginine + I/R group: animals were treated with intravenous L-arginine (300 mg · kg^{-1}) 30 min before ischemia and an intraperitoneal bolus (300 mg · kg^{-1}) at reperfusion.
2. L-Arginine + RPC group: animals injected with L-arginine as per group 1 and then remifentanil 1 μg · kg^{-1} bolus followed by 15 min infusion at 2 μg · kg^{-1} · min^{-1}, finishing 10 min before ischemia.
3. L-NAME + I/R group: animals were treated twice with intravenous L-NAME (30 mg · kg^{-1}) 30 min before ischemia and an intraperitoneal bolus at reperfusion.
4. L-NAME +RPC group: animals were given L-NAME as in group 3, and then remifentanil 1 μg · kg^{-1}bolus followed by 15 min infusion at 2 μg · kg^{-1} · min^{-1}, finishing 10 min before the I/R procedure.

Moreover, to investigate whether ORs mediate the effect of remifentanil pretreatment, naloxone, a nonselective OR antagonist, was used and animals were randomly assigned to one of two groups (n = 10) as follows:

1. Naloxone + I/R group: animals were treated with intravenous naloxone (3 mg · kg^{-1}), (Sigma Chemical Company), 30 min before ischemia.
2. Naloxone + RPC group: animals injected with naloxone as per group 1 and then remifentanil 1 μg · kg^{-1}

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bolus followed by 15 min infusion at 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$, finishing 10 min before the I/R procedure.

All of these animals subsequently underwent the same I/R procedure as in the first series of experiments.

Measurement of Serum Aminotransferase, Tumor Necrosis Factor-α, Interleukin-1 Concentrations, and Nitric Oxide Production. Serum alanine aminotransferase and aspartate aminotransferase were measured using a commercially available kit (Nanjing Jiancheng Biochemicals Ltd., Nanjing, China) with a Model 7600 autobiochemistry analyzer (Hitachi, Tokyo, Japan). Nitrite and nitrate (NOx) content were measured to estimate the total amount of nitric oxide production. The serum was filtered through a 10-kDa molecular mass cutoff filter to eliminate any proteins. The total nitrite concentration was measured using a total nitric oxide assay kit (Nanjing Jiancheng Biochemicals Ltd.) and its detection range was 0–320 $\mu mol \cdot ml^{-1}$ each with a sensitivity of 0.1 $\mu mol \cdot ml^{-1}$. Serum cytokine, tumor necrosis factor-α and interleukin-1 (IL-1), concentrations were measured by enzyme-linked immunosorbent assay kits (Boshide Biochemicals Ltd., Wuhan, China) and the detection range was 0–320 $pg \cdot ml^{-1}$ each with a sensitivity of 1 pg/ml.

Lipid Peroxidation Determination, Superoxide Dismutase, and Myeloperoxidase Activity Assay. Liver homogenates were prepared by immediate centrifugation at 8,000 *g* for 10 min, and hepatic tissue change of malondialdehyde, superoxide dismutase, and myeloperoxidase were assessed by biochemical kits purchased commercially (Nanjing Jiancheng Biochemicals Ltd.). The detection range was 0–200 $mmol \cdot l^{-1}$ for malondialdehyde, 0–800 $U \cdot ml^{-1}$ for superoxide dismutase, and 0–100 $U \cdot ml^{-1}$ for myeloperoxidase.

Histopathology Examination and Apoptotic Cell Detection by Terminal Deoxynucleotide Transferase-mediated Deoxyuridine Triphosphate Nick-end Labeling. All the histopathology and immunohistology examinations were performed by a blinded pathologist to identify underlying necrosis and apoptosis status or other types of injury. Liver samples were excised from the anterior edge of the left lobe 120 min after reperfusion. Small portions (0.5 cm $\times$ 0.5 cm) were fixed immediately in 10% buffered paraformaldehyde (pH 7.2) and embedded in paraffin. These portions were cut into 4-μm-thick sections and stained with hematoxylin and eosin. High-powered microscopy (1 $\times$ 200) was used to examine these sections for the following signs of liver injury: condensation of nuclei (nuclear pyknosis), loss of hepatocellular borders, and areas of necrosis. The percentage was determined by dividing the measured necrotic area by the total area of the field using Image-Pro-Plus® Software (Media Cybernetics Inc., Bethesda, MD). All groups of paraffin sections of liver biopsies were examined for apoptosis using the transferase-mediated deoxyuridine triphosphate nick-end labeling method (*In Situ* Cell Detection Kit, Roche Biochemicals, Mannheim, Germany). The number of terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling positive cells was counted in six random high-powered (1 $\times$ 400) microscopic fields and the apoptosis index was defined as the number of apoptotic cells in every 100 counted cells using Image-Pro-Plus® Software.

Immunohistology of iNOS and Endothelial NOS in Liver Tissue. The fixed liver block was embedded in paraffin and sectioned into 5-μm slices. After deparaffinage and rehydration, the sections were dipped into an antigen retrieval buffer (10 mM sodium citrate; pH 6.0) and underwent warm retrieval in a microwave oven at 95–100°C for 5 min and then incubated at room temperature with 3% hydrogen peroxide to deactivate endogenous peroxidases. Nonspecific reactivity was blocked using 2% bovine serum albumin at room temperature for 30 min. This was followed by incubating the primary antibody rabbit–anti-rat to iNOS or endothelial NOS (eNOS) antibody (dilution 1:100; Abcam Inc., San Francisco, CA), overnight at 4°C. After washing the sections with phosphate-buffered saline, a polymer enhancer and a polymerized antirabbit or antimouse immunoglobulin G (dilution 1:200, Jingmei, Shanghai, China) labeled with horseradish peroxidase was applied. Antibodies were seen as buffy granules in the cytoplasm using a DAB kit (Maixin Biologic Technology, Fujian, China). Area density of NOS-positive tissues was analyzed in six random high-powered (1 $\times$ 400) microscopic fields using Image-Pro-Plus® Software.

Hypoxia/Reoxygenation Injury in Hepatocytes

Hepatocyte Isolation and Culture. Hepatocytes were isolated from 1-day-fasted male Sprague-Dawley rats by the collagenase perfusion method as described previously.[11] Cell viability was greater than 90%, as determined by trypan blue exclusion. The cells were resuspended in Waymouth MB-752/1 medium containing 100 units/ml penicillin, 10% fetal calf serum, 100 nM insulin, and 100 nM dexamethasone.

Hypoxia/Reoxygenation of Cultured Rat Hepatocytes. Hepatocytes were incubated in an anaerobic chamber (Thermo Forma Inc., Marietta, OH) for 4 h. Atmosphere in the anaerobic chamber was kept anoxic with 2% oxygen/93% nitrogen/5% carbon dioxide. This degree and timing of hypoxia was chosen based on established protocols for morphine-induced hepatoprotection.[11] To simulate reoxygenation and recovery to normal intracellular pH after reperfusion, hepatocytes were reoxygenated for up to 2 h at 37°C.

Study Groups and Experimental Protocol *In Vitro*. To determine whether the administration of remifentanil limits hepatocyte HR injury, cells were randomly assigned to receive different concentrations (0.1, 1, 10, 100 ng/ml) of remifentanil pretreatment 10 min before HR injury, Subsequently, to test whether NOS and ORs mediate the effect of remifentanil preconditioning, cells were randomly assigned to one of nine groups as shown in figure 1B: in the sham group, cells were incubated in KRH buffer at pH 6.2 in normal oxygen concentration (21% oxygen/74% nitrogen/5% carbon dioxide); in the HR control group, cells were incubated for 4 h of hypoxia followed by 2 h of reoxygenation; in the RPC group, 10 ng/ml remifentanil pretreat-

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ment was given 10 min before HR; in the naloxone + RPC group (NLX/RPC), 10 μM naloxone was given 30 min before RPC and HRI; In the naloxone control group (NLX), 10 μM naloxone was given 30 min before HRI; in the L-arginine + RPC group (L-arg/RPC), 50 μM L-arginine was given 30 min before RPC and HRI; in the L-arginine control group (L-arg), 50 μM L-arginine was given 30 min before HRI; in the L-NAME + RPC group (L-NAME/RPC), 10 μM L-arginine was given 30 min before RPC and HRI; and in the L-NAME control group (L-NAME), 10 μM L-arginine was given 30 min before HR injury.

Cell Viability Assay. Cell viability was determined using WST-8 dye (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Briefly, 10^5 cells/well were seeded in a 96-well flat-bottomed plate, grown at 37°C for 24 h, and then placed in serum-starved conditions for an additional 6 h. After 10 μl WST-8 dye was added to each well, cells were incubated at 37°C for 2 h and the absorbance was finally determined at 450 nm using a microplate reader.

Intracellular Nitric Oxide Detection. The intracellular nitric oxide concentration was measured using a nitric oxide-sensitive fluorescence probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF FM DA) (Beyotime Institute of Biotechnology). Briefly, cells were loaded with DAF-FM DA (10 μM) at 37°C for 30 min in Krebs-Ringer solution (100 mM NaCl, 2.6 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 11 mM glucose). Then cells were gently washed three times and incubated for an additional 30 min to ensure complete cleavage of DAF-FM DA by the intracellular ester enzyme to release the nitric oxide-sensitive probe (DAF-FM). Fluorescence was detected with a fluorescence microscope (FV 500, Olympus, Japan). The digital images were analyzed with Image-Pro-Plus® Software and the average fluorescence density of intracellular areas was measured to index the nitric oxide concentration.

Morphologic Studies of Apoptotic Cells. Hepatocytes were seeded in a 96-well microplate and incubated for 24 h. Next, cells were treated with remifentanil and H/R. After treatment, cells were washed once with phosphate-buffered saline. Cells were then stained with Hoechst 33342 dye assay (Beyotime Institute of Biotechnology) for 15 min at room temperature before examination under a fluorescent microscope with an excitation wavelength of 365 nm. The percentage of apoptosis-positive cells was counted in six random microscopic fields using Image-Pro-Plus® Software.

Western Blot Analysis of iNOS and eNOS in Hepatocytes. iNOS and eNOS protein expression was assessed by Western blot analysis. Cells were homogenized in 0.5 ml detergent buffer containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany). The protein extract from these cells was separated on a 7.5% polyacrylamide gel. Proteins were then transferred onto nitrocellulose, blocked for 1 h at room temperature with 5% milk + 0.05% Tween-20 and incubated with rabbit-antirat to iNOS or eNOS antibody (Abcam Inc.) at 1:1,000 concentration for another hour at room temperature in 5% milk + 0.05% Tween-20. After three washes with phosphate-buffered saline + 0.05% Tween-20, the blots were incubated with peroxidase-conjugated goat anti-rat immunoglobulin G (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:15,000 dilution for 1 h at room temperature in 5% milk + 0.05% Tween 20. Membranes were again washed as discussed previously and then incubated with ECL Plus (Amersham Pharmacia, Piscataway, NJ) and visualized on film. Blots were quantified using Image-Pro-Plus® Software.

Statistical Analysis

Data analysis was performed with a personal computer statistical software package (Prism version 4.0; Graph-Pad Software, San Diego, CA). Data are expressed as mean ± SD. One-way ANOVA and the Student-Newman-Keuls q test were used to compare values among all groups. Serum enzymes and peroxidation and cytokines variables were analyzed using one- way ANOVA with Student–Newman–Keuls q test. Statistical differences were considered significant if the P value was less than 0.05. All P values are results of two-sided tests.

Results

A total of 117 animals were used in the *in vivo* study. Four animals were excluded from further experimentation before assignment to treatment groups because of severe blood loss during portal vein exposure, and three in the I/R group were excluded because of excessive ischemia and consequent liver damage after reperfusion (possible portal thrombosis). A total of 110 animals completed the *in vivo* study.

Effect of Remifentanil on Attenuating Liver I/R Injury

Serum alanine aminotransferase and aspartate aminotransferase concentrations were significantly decreased 2 h after reperfusion in the RPC2 and RPC3 groups when compared with the I/R group. There was no significant difference in aminotransferase concentrations between the RPC1 and I/R group (table 1). At 120 min after reperfusion, the liver histology of all remifentanil-pretreated groups exhibited fewer and smaller areas of necrosis, sinusoidal dilation, and structural derangement around the central vein in comparison with the I/R group. The amount of apoptosis and the number of terminal deoxynucleotidyltransferase deoxyuridine triphosphate nick-end labeling positive cells among RPC2 and RPC3 groups were less than that in the I/R groups (fig. 2).

Remifentanil Pretreatment Reduced Lipid Peroxidation and Inflammation in Rat Liver I/R Injury

At 120 min after reperfusion, rats pretreated with remifentanil had significantly decreased concentrations of malondial-

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Table 1. Serum Aminotransferases, Cytokines, Nitric Oxide Concentrations, Liver Lipid Peroxdation, and Myeloperoxidase Activity When Pretreated with Different Dosages of Remifentanil

	Sham	IR	RPC1	RPC2	RPC3
ALT ($IU \cdot l^{-1}$)	34 ± 18*	336 ± 91	278 ± 105	77 ± 41*	90 ± 45*
AST ($IU \cdot l^{-1}$)	30 ± 21*	399 ± 101	328 ± 127	148 ± 105*	158 ± 90*
TNFa ($pg \cdot ml^{-1}$)	28 ± 5*	94 ± 12	85 ± 10	53 ± 8*	50 ± 7*
IL1 ($pg \cdot ml^{-1}$)	26 ± 3*	184 ± 21	157 ± 18	90 ± 8*	82 ± 12*
NO_X ($\mu mol \cdot ml^{-1}$)	9.9 ± 2.1*	3.7 ± 1.9	5.1 ± 3.8	31.2 ± 4.8*	33.9 ± 6.1*
MDA ($\mu mol \cdot l^{-1}$)	4.1 ± 1.56*	19.3 ± 2.2	17.1 ± 1.3	9.1 ± 1.1*	9.7 ± 1.9*
SOD ($U \cdot ml^{-1}$)	69.4 ± 12.1*	56.1 ± 5.0	67.3 ± 10.5	89.1 ± 8.3*	90.2 ± 9.3*
MPO ($U \cdot g\ tissue^{-1}$)	0.5 ± 0.2*	1.9 ± 0.3	1.7 ± 0.4	0.7 ± 0.3*	0.7 ± 0.4*

Values are mean ± SD of 10 animals in each group.

* $P < 0.05$ *vs.* IR group value.

ALT = alanine aminotransferase; AST = aspartate aminotransferase; IL1 = interleukin-1; IR = ischemia-reperfusion injury; MDA = malondialdehyde; MPO = myeloperoxidase; NO= nitric oxide; NO_X = nitrite and nitrate; RPC1, RPC2, RPC3 = remifentanil infusion of 0.2, 2, and 20 $\mu g \cdot kg^{-1} \cdot min^{-1}$; SOD = superoxide dismutase; TNFa = tumor necrosis factor-a.

dehyde and myeloperoxidase activity whereas superoxide dismutase concentrations in liver homogenates were increased. Serum tumor necrosis factor α and interleukin-1 concentrations decreased significantly in RPC2 and RPC3 groups, but not in the RPC1 group, in comparison with I/R (table 1).

Effect of Nitric Oxide and NOS During Remifentanil Preconditioning in Liver Subjected to I/R

Serum alanine aminotransferase and aspartate aminotransferase concentrations in rats pretreated with L-arginine and remifentanil were significantly lower than those in I/R. The

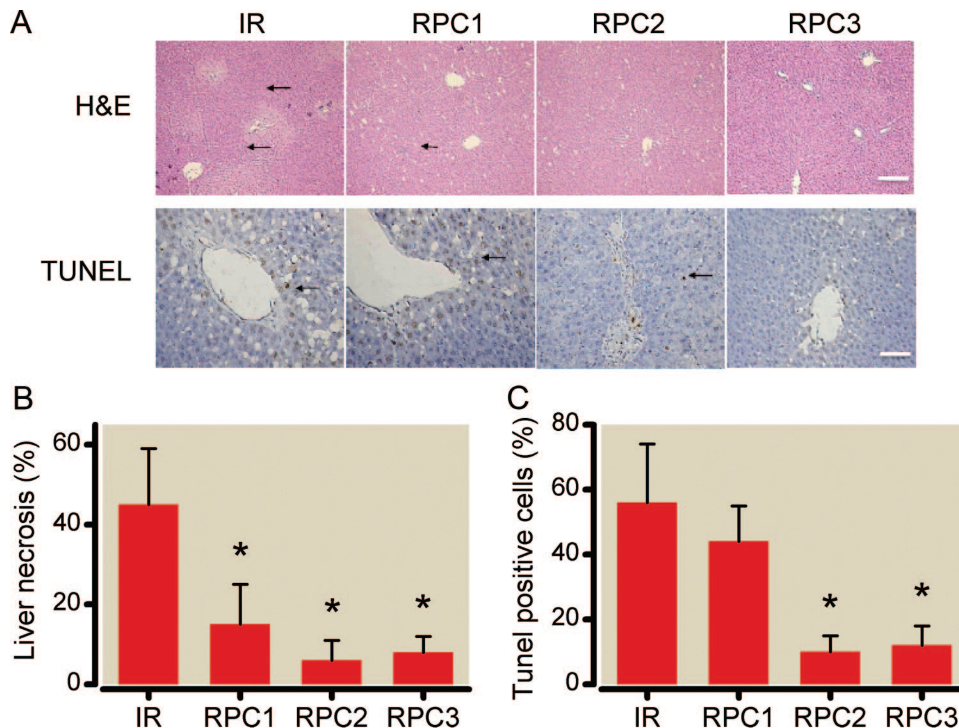

Fig. 2. Effect of remifentanil preconditioning on liver ischemia reperfusion injury (n = 10 in each group). (*A*) Hepatic tissue histologic changes were processed with hematoxylin and eosin (H&E) staining for light microscopy examination. Photograph depicts typical pattern of focal necrosis (*black arrows*) after ischemic degeneration of hepatocytes around the central venous area. Hepatocyte apoptosis was determined by immunohistochemical analysis by microscopy after terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining. Photograph depicts typical pattern of apoptotic cells (*black arrows*). (*B*) Areas of necrosis were significantly decreased in all remifentanil-pretreated groups than in ischemia-reperfusion group (IR) (* $P < 0.05$ *vs.* IR). Magnification: 200×. Scale bar = 200 μm. (*C*) Percentage of apoptotic cells was significantly lower in 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ remifentanil preconditioning (RPC2) and 20 $\mu g \cdot kg^{-1} \cdot min^{-1}$ remifentanil preconditioning (RPC3) groups while not in 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ remifentanil preconditioning (RPC1) group than in IR. Magnification: 400×. Scale bar = 100 μm (* $P < 0.05$ *vs.* IR).

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Table 2. Serum Aminotransferases and NO Concentrations When Using NOS Inhibiter or NO Donor before Remifentanil Pretreatment

	IR	RPC	L-arg/IR	L-arg/RPC	L-NAME/IR	L-NAME/RPC
ALT (IU · l^{-1})	336 ± 91	77 ± 41	178 ± 69*	134 ± 37*	677 ± 129*	628 ± 170*
AST (IU · l^{-1})	399 ± 101	148 ± 105	290 ± 67*	225 ± 44*	844 ± 312*	728 ± 99*
NO_X (μmol · ml^{-1})	3.7 ± 1.9	31.2 ± 4.9	32.1 ± 6.2*	41.9 ± 5.2*	7.0 ± 3.9	10.1 ± 3.5

Values are mean ± SD of 10 animals in each group.

* $P < 0.05$ *vs.* IR group value.

ALT = alanine aminotransferase; AST = aspartate aminotransferase; IR = ischemia-reperfusion injury; L-arg = a nitric oxide donor (L-arginine); L-NAME = *N*-w-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase; NO = nitric oxide; NO_X = nitrite and nitrate; RPC = 2 μg · kg^{-1} · min^{-1}.

two groups with L-NAME pretreatment had significantly increased aminotransferases concentrations compared with I/R (table 2). Serum NOx concentrations were higher in L-arginine groups than in I/R- and L-NAME-pretreated groups (table 2).

At 120 min after reperfusion, L-arginine groups exhibited much less necrosis, sinusoid dilation, and sinusoid neutrophil accumulation and less structural derangement in histology around the central vein in comparison with those in I/R. L-NAME-pretreated groups showed even more severe histologic injury than I/R (fig. 3). The degree of apoptosis and the number of terminal deoxynucleotidyltransferase deoxyuridine triphosphate nick-end labeling positive cells in the L-arginine pretreated groups was also decreased (fig. 3). Immunohistology (fig. 4) showed an increase in iNOS but not eNOS protein expression in RPC and L-arginine-pretreated groups compared with I/R and L-NAME groups.

Role of Opioid Receptors in Remifentanil Preconditioning in Rat Livers Subjected to I/R

There was no difference in alanine aminotransferase and aspartate aminotransferase concentrations between naloxone and the I/R group. Alanine aminotransferase and aspartate aminotransferase concentrations in groups pretreated with naloxone and remifentanil were significantly lower than those in the I/R group (table 3).

Effect of Remifentanil on Attenuating HR Injury in Hepatocytes

Cell viability decreased after 4 h of hypoxia and 2 h of reoxygenation. In contrast, administration of remifentanil to hepatocytes for 10 min before anoxia substantially reduced HR-induced cell death. This was a dose-dependent effect because remifentanil 0.1 ng · ml^{-1} did not prevent cell death, whereas concentrations greater than 1 ng · ml^{-1} improved

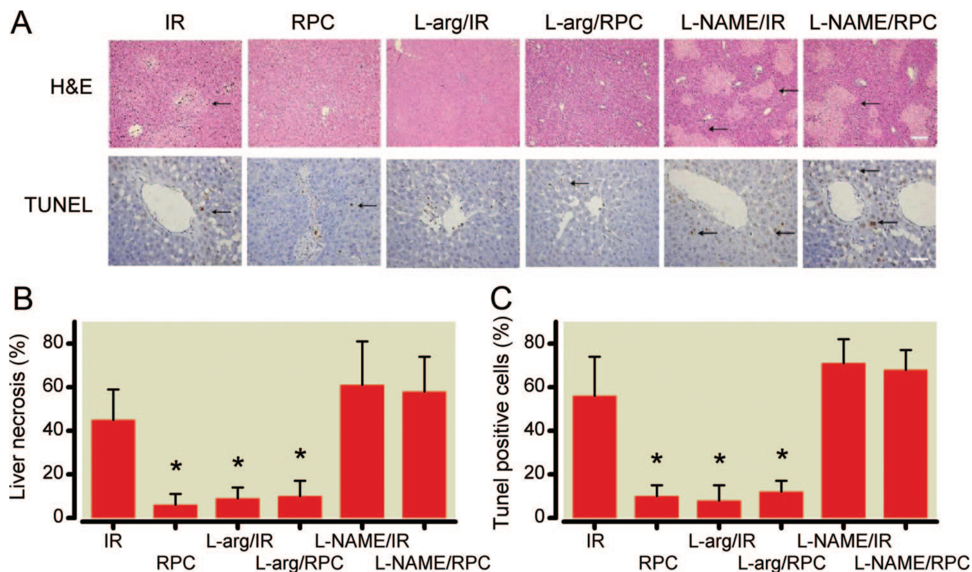

Fig. 3. Effects of nitric oxide donor and nitric oxide synthase inhibitor on hepatic histology and apoptosis (n = 10 in each group). (*A* and *B*) Photograph depicts typical pattern of focal necrosis after ischemic insult. Black arrows indicate ischemic changes that were seen more seriously in *N*-ω-nitro-L-arginine methyl ester (L-NAME) treatment and ischemia-reperfusion (IR) groups compared with other groups. Magnification: 200×. Scale bar = 200 μm (* $P < 0.05$ *vs.* IR). (*A* and *C*) The degree of apoptosis and the number of terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) positive cells (black arrows) were decreased in 2 μg · kg^{-1} · min^{-1} remifentanil preconditioning (RPC) and L-arginine (L-arg) pretreated groups. Magnification: 400×. Scale bar = 100 μm (* $P < 0.05$ *vs.* IR). H&E = hematoxylin and eosin.

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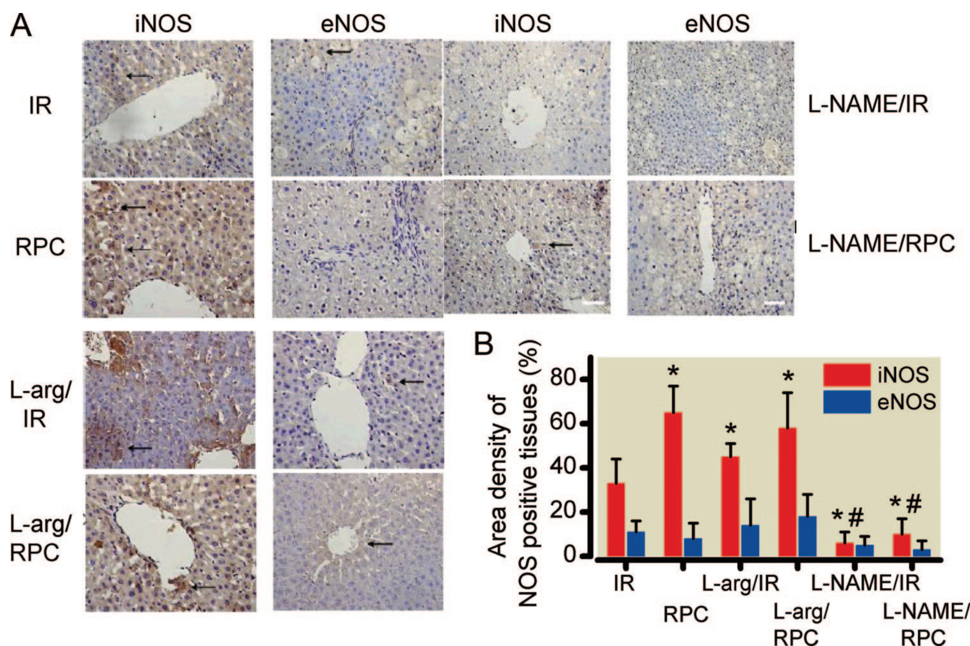

Fig. 4. Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) protein expression in various groups. (*A*) Hepatic iNOS and eNOS positive cells were defined as stained with brown in cytoplasm (*black arrows*). (*B*) There was an increase in iNOS but not eNOS expression in remifentanil preconditioning (RPC) and L-arginine (L-arg) pretreated groups compared with ischemia-reperfusion (IR), whereas it also showed a decrease in iNOS but not eNOS protein expression in *N*-ω-nitro-L-arginine methyl ester (L-NAME) pretreated groups compared with RPC magnification: 400×. Scale bar = 100 μm (* $P < 0.05$ *vs*. IR; # $P < 0.05$ *vs*. RPC).

cell viability. Consequently, a 10 ng · ml^{-1} remifentanil concentration was used for our subsequent research (fig. 5). Hepatocyte viability decreased significantly after HR injury. In contrast, cells receiving remifentanil and L-arginine pretreatment had reduced HR-induced cell death. The effect was prevented by coadministration of L-NAME with or without remifentanil, and pretreatment with naloxone could not block the protective effect of remifentanil. There was no significant change in cell viability between naloxone alone and control groups (fig. 6). Intracellular nitric oxide concentration increased and the cells' apoptotic ratios decreased significantly in remifentanil and L-arginine pretreated groups compared with control groups, with these effects prevented by coadministration of L-NAME but not naloxone (figs. 5 and 6). Western blot analysis (fig. 7) showed an increase in iNOS but not eNOS protein expression in remifentanil, naloxone plus remifentanil, and L-arginine pretreated groups compared with control and L-NAME groups.

Table 3. Serum Aminotransferases Concentrations in Naloxone Blocking Experiment

	IR	RPC	NLX/RPC	NLX/IR
ALT (IU · l^{-1})	336 ± 91	78 ± 41*	97 ± 56*	345 ± 113
AST (IU · l^{-1})	399 ± 101	148 ± 105*	176 ± 92*	479 ± 173

Values are mean ± SD.

* $P < 0.05$ *vs.* IR group value of 10 animals in each group.

ALT = alanine aminotransferase; AST = aspartate aminotransferase; IR = ischemia-reperfusion injury; NLX = naloxone; RPC = 2 μg · kg^{-1} · min^{-1}.

Discussion

Forty-five minutes of ischemia followed by 2 h reperfusion results in liver injury with higher concentrations of serum aminotransferase, cytokines, and hepatic lipid peroxidation as well as histologic changes and apoptosis. Because each intervention had its own control group and no damage was seen in the sham group, we can assume that the lesions in the liver were caused by ischemia. The current study, therefore, demonstrates the beneficial effects of remifentanil pretreatment on acute hepatic I/R injury in both intact rats and in isolated hepatocytes with HR injury. These protective effects were accompanied by increased concentrations of nitric oxide and the expression of iNOS. The beneficial effects were blocked by the previous administration of a NOS inhibitor but not with an OR antagonist both *in vivo* and *in vitro*. Remifentanil appears to precondition the liver against I/R injury by decreasing lipid peroxidation and inflammation, as evident by decreased concentrations of malondialdehyde, myeloperoxidase, tumor necrosis factor α and interleukin-1 and increased concentrations of superoxide dismutase in remifentanil-treated groups. These results suggest that iNOS might partly mediate this effect by exhausting reactive oxygen species and attenuating the inflammatory response through generation of endogenous nitric oxide.

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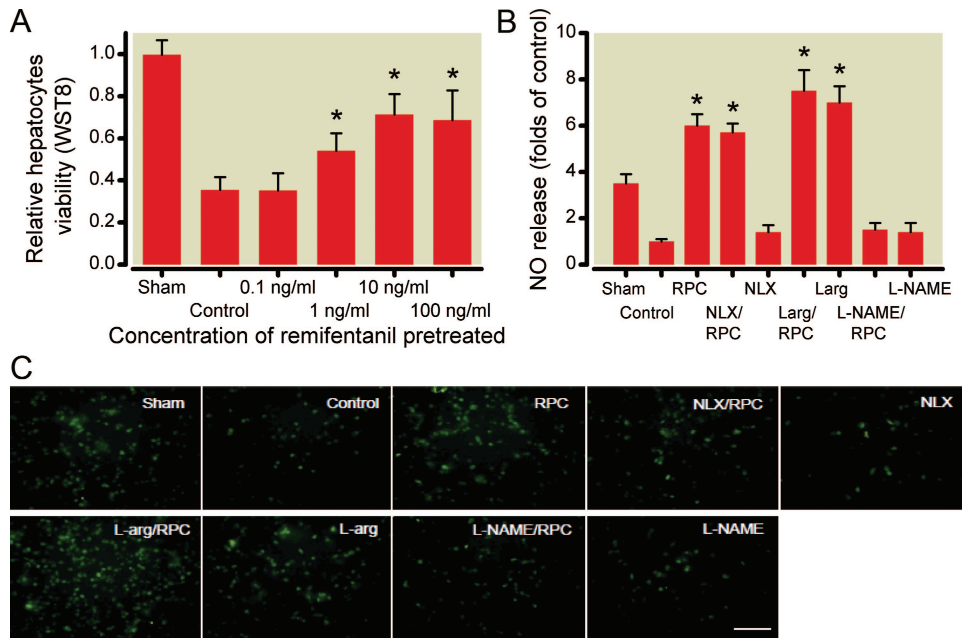

Fig. 5. Effect of remifentanil on hepatocyte viability and nitric oxide release after hypoxia reoxygenation injury. (*A*) Hepatocytes viability measured by WST-8 dye decreased significantly after ischemia-reperfusion injury, whereas the pretreatment of remifentanil 1 ng · ml^{-1} or higher produced a protective effect *in vitro* (* $P < 0.05$ *vs*. Control). (*B* and *C*) Intracellular nitric oxide concentration was measured by *in situ* by DAF-FM diacetate, and shown by the fluorescence in green color. Hepatocytes nitric oxide increased significantly in animals pretreated with remifentanil or L-arginine (L-arg) compared with control, while these effects were prevented by coadministration of *N*-ω-nitro-L-arginine methyl ester (L-NAME) but not opioid antagonist (naloxone) before remifentanil preconditioning (RPC). Magnification: 200×. Scale bar = 200 μm (* $P < 0.05$ *vs*. Control).

Our method of partial I/R injury rather than total inflow occlusion in the rat liver has been extensively used in previous studies, and 45 min of ischemia followed by 2 h of reperfusion might also result in moderate and remediable liver dysfunction as reported.[12] We measured a range of histologic and biochemical variables to provide a more complete picture of hepatic injury. In the early stages of hepatic reperfusion, an imbalance between endothelin and nitric oxide concentrations results in failure of the hepatic microcirculation. Reperfusion promotes proinflammatory cytokine and adhesion molecule synthesis, resulting in oxygen-derived free radical production and neutrophil recruitment that further contribute to cellular injury.[13] Necrotic cell death is the final consequence of microvascular I/R injury leading to dysfunction or primary nonfunction of the postischemic liver, whereas apoptosis has been identified as another important mechanism leading to organ dysfunction.[2] To determine the role of iNOS we also examined the effects of remifentanil pretreatment in isolated hepatocytes. HR-induced oxidative stress and cell damage *in vitro* partly reflect I/R injury *in vivo*.[14,15] In addition to measuring serum transaminase concentrations, which have been widely accepted as a marker of hepatocyte injury, we also measured lipid peroxidation in the postischemic liver tissue in an attempt to quantify oxidative stress.

There is strong evidence of the ability of opioids to reduce I/R injury of the myocardium[6,16] and nerve cells.[7] Remifentanil has unique pharmacokinetic advantages over morphine that allow it to be given in very high doses intraoperatively without fear of prolonged effects such as ventilatory depression.[17,18] It has been studied most extensively in the heart where the *in vivo* protection is mediated *via* δ, κ, and μ ORs, although the drug is predominantly a μ agonist.[17,19] Recently, it has also been demonstrated that remifentanil can suppress inflammatory response and reactive oxygen species production in mice with sepsis and conferred a protective effect against sepsis.[8] From a clinical perspective, remifentanil could be a particularly attractive preconditioning agent in liver surgery and transplantation because it has a short half-life that is unaffected by liver function.[17] An *in vivo* study has shown that the opioid peptide DADLE attenuated liver I/R injury *via* an antioxidative effect,[20] and other studies have shown that inflammatory pathway toll-like receptor signaling is also involved in opioid-induced protection in the brain and cell cultures.[21,22] Opioid agonists have an inhibitory effect on proinflammatory cytokine production in human monocytes after toll-like receptor 2 stimulation, and this inhibition is mediated solely by μ-ORs.[23] Toll-like receptor signaling also plays a key role in the regulation of inflammation during hepatic I/R by releasing proinflammatory cytokines such as tumor necrosis factor α and interleukin-1.[24] Our data show that pretreatment with remifentanil decreases serum cytokine concentrations as well as decreases the accumulation of neutrophils in ischemic hepatic tissue, which is consistent with these findings.

The mechanisms underlying opioid-induced hepatic protection may be multifactorial.[7,25] It is well known that endogenous nitric oxide may act to limit reactive oxygen species

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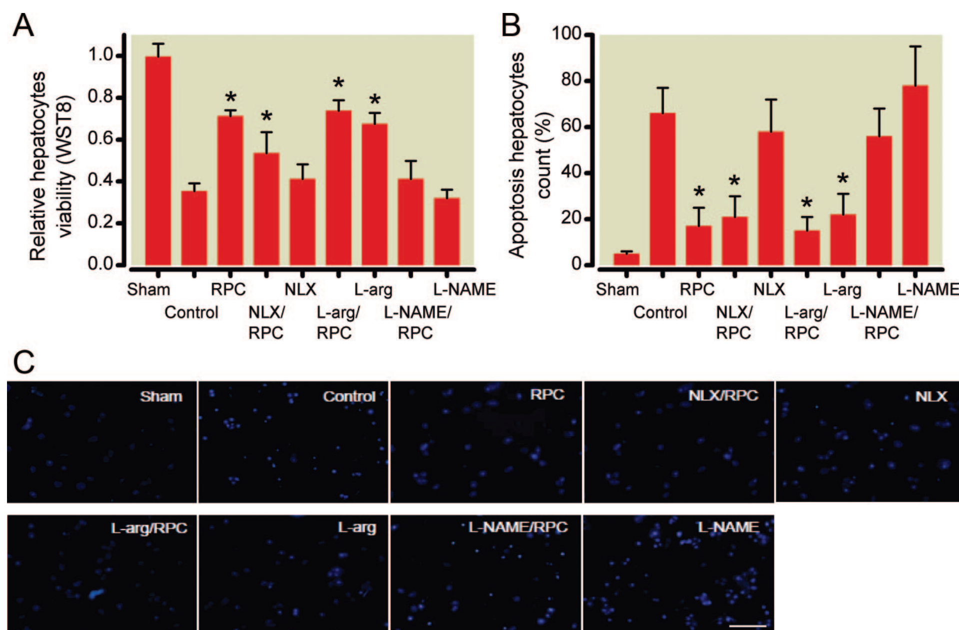

Fig. 6. Effects of nitric oxide and naloxone on remifentanil pretreatment of hepatocytes viability and apoptosis by hypoxia reoxygenation. (*A*) Cells in remifentanil (10 ng · ml^{-1}) and L-arginine (L-arg) pretreatment reduced reoxygenation-induced cell killing, measured by WST-8 dye, whereas the effect was prevented by coadministration of *N*-ω-nitro-L-arginine methyl ester (L-NAME) with or without remifentanil. Pretreatment with naloxone (NLX) could not block the effect of remifentanil preconditioning (RPC) (* $P < 0.05$ *vs*. Control). (*B* and *C*) Hepatocytes apoptosis was assessed using *in situ* Hoechst 33342 staining. Normal nuclei show faint delicate chromatin stain, with the nuclei at the early stage of apoptosis displaying an increased condensation and brightness. Percentage of cells apoptosis decreased significantly in remifentanil or L-arginine pretreated groups compared with the control group, whereas these effects were prevented by coadministration of L-NAME but not opioid antagonist (naloxone) before remifentanil preconditioning. Magnification: 200×. Scale bar = 200 μm (* $P < 0.05$ *vs*. Control).

and polymorphic neutrophil-mediated tissue injury as well as attenuating the subsequent inflammatory response.[13] It has also been demonstrated that I/R-induced cellular injury is associated with a marked decrease in the bioavailability of nitric oxide, which is an important initiating event in the pathophysiology of postischemic injury in a variety of tissues.[26,27] The protective effect of the nitric oxide donor L-arginine against I/R injury in hepatocytes is partially due to production of nitric oxide and by increased vasodilatation.[28] In the liver, constitutively generated nitric oxide maintains the hepatic microcirculation and endothelial integrity, whereas nitric oxide synthases governing nitric oxide production can be either beneficial or detrimental.[29,30] There is evidence that adenovirus iNOS pretreatment is protective and that Kupffer cells also protect the liver *via* an iNOS-dependent means.[31,32] These mechanisms might also partly explain the results of the current study because remifentanil pretreatment not only ameliorated hepatic lipid peroxidation indices but also decreased serum cytokine concentrations accompanied by increased protein expression of iNOS and serum nitric oxide concentration. This then resulted in a reduction in hepatic histologic injury and inhibited hepatic cellular apoptosis. The results also suggest that iNOS contributed to the attenuation of warm I/R injury by limiting reactive oxygen species and polymorphic neutrophil-mediated tissue injury.

Although our observation that the increased expression of iNOS rather than eNOS by remifentanil preconditioning seems to support the beneficial role of iNOS in hepatic I/R injury as previously reported,[32] the relative contribution of each isoform in liver injury after reperfusion is much less clear. Wang *et al.*[33] had reported that the selective iNOS inhibitor 2-aminoethyl-isothiourea increased hepatic damage in a model combining endotoxemia and I/R. However, there is other evidence showing that iNOS may contribute to hepatic injury, whereas eNOS is protective and seems to play a crucial role after ischemia.[34] We think this may be due to different hepatic I/R protocols and timing of detection. Using iNOS knockout mice, it was shown that iNOS absence was associated with increased plasma transaminase concentrations than in wild-type mice as early as 3 h after reperfusion, suggesting an early iNOS-dependent contribution to intrahepatic perfusion after ischemia.[35] In our study, we measured the histologic and biochemical markers after 2 h of reperfusion, so the superior remifentanil pretreatment effects might be related to this early iNOS-dependent benefit.

Although by using naloxone both in animal and in cell culture we demonstrated that remifentanil's beneficial effects were OR-independent, our results contrast with some opioid antagonist studies in sepsis[36] and cholestatic liver disease.[37] Other studies have demonstrated that opioid antagonists relieved Fas-induced hepatocyte apoptosis through modu-

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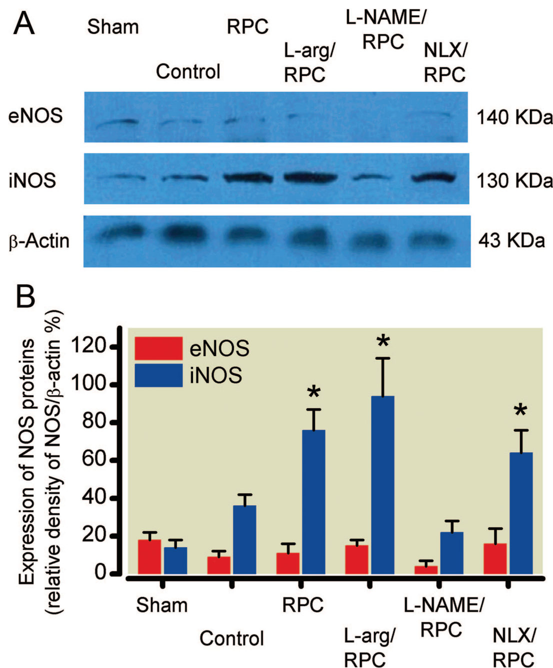

Fig. 7. Western blot analysis of iNOS and eNOS in hepatocytes. (*A* and *B*) Western blot analysis showed an increase in iNOS protein expression but not in eNOS in the groups of remifentanil, naloxone plus remifentanil, and L-arginine (* $P <$ 0.05 *vs.* Control). eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase; L-arg = L-arginine; L-NAME = *N*-ω-nitro-L-arginine methyl ester; NLX = naloxone; RPC = remifentanil preconditioning.

lation of nonparenchymal cells[38] and that increased concentrations of endogenous opioids contribute to hepatocyte apoptosis in cholestasis.[39] These contradictory data could be explained by the administration of different doses, different timing as well as different types of hepatic injury. Although it has been suggested that high doses of or chronic exposure to morphine causes hepatocyte apoptosis by inducing oxidative stress,[40] there is still evidence that endogenous opioids attenuate liver I/R injury *via* an antioxidative effect.[22,41] Chakass *et al.*[42] also proved that μ OR agonists might prevent acute hepatitis and have promising therapeutic use to maintain remission in both chronic inflammatory bowel and liver diseases. We believe the above data support our hypothesis and results, because opioid-induced apoptosis has mostly been shown in cholestasis[43] or lipopolysaccharide-pretreated liver[36] but not in acute I/R injury. It is also likely that the systemic condition and availability of endogenous opioids make a difference with various injury types.[8] Unlike cardiomyocytes, it appears that the hepatocytes lack ORs.[38] In this study we also demonstrated that hepatocellular protection by remifentanil preconditioning could not be blocked by naloxone, suggesting that an opioid-independent mechanism mediates this effect, in accordance with another study.[11]

Our study does have some limitations: (1) remifentanil pretreatment's beneficial effects were observed at doses of more than 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ *in vivo* and a concentration of 1 ng/ml *in vitro*, which is larger than normal clinical use; (2) these effects were observed in rats and not humans, so clinical trials would be needed to better establish its potential role in reducing hepatic I/R injury; (3) we observed these effects in nondiseased livers of young healthy animals and not in diseased animals; advanced age and systemic diseases such diabetes are known to markedly interfere with ischemic preconditioning in other organs.[44,45] Additional studies will be needed to explore remifentanil pretreatment's effects in specific animal models.

In summary, our study suggests that remifentanil pretreatment can confer hepatic protection against both I/R injury *in vivo* and hypoxia reoxygenation injury *in vitro*. iNOS might partly mediate this effect by decreasing the production of reactive oxygen species and limiting the inflammatory response by generating endogenous nitric oxide in hepatocytes.

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