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Urethane suppresses rat lung inducible cyclooxygenase and nitric oxide synthase mRNA levels

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Abstract. *Objective and Design:* The purpose of the present study was to evaluate the effect of urethane, pentobarbital sodium and ketamine-xylazine anesthesia upon constitutive and inducible cyclooxygenase (COX-1; COX-2) and nitric oxide synthase (eNOS; iNOS) mRNA levels in the lung.
Methods: mRNA levels were determined by the semiquantitative RT-PCR technique.
Treatment: Urethane (1.1 g/kg ip), Pentobarbital Sodium (40 mg/kg ip), and ketamine (85 mg/kg) – xylazine (15 mg/kg, im). Non-anesthetized animals served as controls.
Material: Sprague-Dawley rat lungs
Results: Urethane significantly decreased COX-1 and COX-2 mRNA levels to 30% of control values. This agent had no effect upon eNOS, but completely suppressed iNOS mRNA levels. Pentobarbital sodium and ketamine had no effect on the mRNA levels for COX-1 and COX-2 the lung.
Conclusions: Urethane has a suppressive effect on COX and iNOS RNA message in the lung and for this reason it should be avoided as an anesthetic when lung inflammatory processes are experimentally evaluated in the rat.

Key words: Ketamine – Pentobarbital

Introduction

Urethane is an anesthetic agent that although never used in humans, is frequently the drug of choice for acute animal experimentation because it results in deep, surgical level anesthesia with minimal physiological changes of the cardiovascular system. In respiratory related research its usefulness is based on the fact that it provides surgical level analgesia, with no effect on the control of breathing [1–4]. A Medline search of the scientific literature since 1975 uncovered 369 published manuscripts related to lung research in urethane treated animals. In the last decade alone 102 published papers met this criterion. In some of the published studies where urethane anesthesia was utilized, the researchers were interested in evaluating the immune response or inflammatory processes in the lung [5].

Aside from its anesthetic properties, this drug has been shown to induce physiological changes that suggest a powerful anti-inflammatory effect. These changes include fever inhibition [6, 7], inhibition of carrageenan-induced inflammation [8], attenuation of lipopolysaccharide (LPS) induced lung inflammation and leukopenia [9]. It has been postulated that such anti-inflammatory influences of urethane are secondary to a tumor necrosis factor-α suppression [9] or the result of a drug-induced increase in corticosterone and epinephrine levels [10].

Prostaglandin products of the cyclooxygenases are well known to be involved in inflammatory processes. The commonly clinically utilized non-steroidal anti-inflammatory drugs act by inhibiting the cyclooxygenases. There are two forms of the enzyme, a constitutive isoform (COX-1) and an inducible form (COX-2) which is documented to be involved in many inflammatory processes [11].

Nitric oxide is also involved in the changes associated with inflammation. In contrast to the COX system, two constitutive forms of nitric oxide synthase exists (eNOS, nNOS), as well as an inducible form (iNOS) which is also known to be involved in some inflammatory processes [12]. Whether, the anti-inflammatory effect of urethane is related to cyclooxygenase or nitric synthase inhibition has never been previously evaluated.

Recently we utilized urethane anesthesia in a research protocol geared at evaluating the lung COX-2 and iNOS mRNA level changes following different stimuli. Preliminary data suggested that urethane might suppress the mRNA expression of these enzymes. This observation led us to further evaluate this phenomenon in the rat lung.

Therefore, the purpose of the present study was to evaluate the effect of urethane anesthesia on the lung levels of mRNA for cyclooxygenases and nitric oxide synthases in rat. We hypothesized that urethane suppresses lung tissue expression of these enzymes. In order to control for the anesthetic effects of the drug, we also evaluated two other commonly used anesthetics in rats: pentobarbital and ketamine-xylazine.

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Methodology

Adult Sprague Dawley rats were provided deep surgical level anesthesia with one of three drugs: urethane (1.1 g/Kg ip), pentobarbital sodium (40 mg/Kg ip) or ketamine-xylazine (ketamine: 85mg/Kg; xylazine 15 mg/Kg im). Whenever any significant motor activity was observed, pentobarbital sodium and ketamine-xylazine were further administered at a dose 25% of the initial. The animals receiving urethane did not require subsequent doses to maintain anesthesia. While anesthetized, the rats were maintained at a constant room temperature of 20 °C that was monitored with an environmental thermometer. The rats body temperature was monitored with a rectal probe and did not change during anesthesia in any of the groups. Following 6 hours of anesthesia the rats were killed with an intracardiac overdose of pentobarbital sodium (50 mg/Kg). Non-anesthetized animals were killed with a similar dose of pentobarbital sodium ip and served as controls. After death, the chest was opened, the lungs were flushed free of blood by perfusing the main pulmonary artery with normal saline and then removed. Five animals were studied for each drug and another five served as controls.

The lung tissue was collected, washed in saline, blotted on filter paper, weighed, frozen in liquid nitrogen, and stored at −70 °C prior to RNA extraction. The University of Calgary Animal Care Committee approved the protocol for this study and the animals were maintained in accordance with Canadian Council on Animal Care guidelines.

RNA Isolation

Total RNA was isolated from frozen tissues as described previously [13, 14]. Briefly, frozen tissue (50–100 mg) was reduced to a powder in a liquid nitrogen-cooled Braun Mikro-Dismembrator Vessel (B. Braun Biotech International; Allentown, PA, USA) and their RNA content extracted. The yield of RNA was quantified spectrophotometrically (UltroSpec 3000 UV/VIS Spectrophotometer; Pharmacia Biotech, Cambridge, England, UK), and optical density (OD) 260/OD 280 ratios were determined.

Lung tissue COX-1, COX-2, eNOS and iNOS mRNA levels were assessed using the semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) duplex technique. The following semiquantitative RT-PCR method was used. 1 µg of RNA from each sample was reverse transcribed at 42 °C for 50 min using 1 µL (200 units) Superscript Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD) and the appropriate reaction mixture containing 2 µL of 10X PCR buffer, 2 µL of 10 mmol/L deoxynucleoside triphosphate [dNTP] stock, and 2 µL of N6 random hexamer stock. DNA amplification was conducted under the following conditions: denaturation at 94 °C for 1 minute, annealing at 58 °C for 30 seconds, and extension at 72 °C for 1 minute. At the end of procedure, a final extension at 72 °C for 7 min was performed. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as an internal control. PCR products were visualized by electrophoresis of 20 µL of reaction mixture on a 2% agarose gel at 60 V/cm in TAE buffer (0.4 M Tris-acetate, 0.001 M EDTA, pH 8.0). In order to ensure that GAPDH mRNA levels are not affected by the anesthetic drugs evaluated, mRNA levels were compared with another housekeeping gene (β-actin). Table 1 list the sequences and number of PCR cycles for the primer sets used in this study. All no-RT controls were negative, indicating no detectable genomic DNA contamination in the samples.

The gels were stained with ethidium bromide, and destained with distilled water. The gels were then photographed using Polaroid Type 55 land film, and analyzed by densitometry (MasterScan Interpretive Densitometer and RFLPscan, Scanalytics, CSPI), as previously described [13, 14]. Integrated density values were normalized to GAPDH values to yield a semi-quantitative assessment of individual transcript levels, which are presented in the figures as normalized integrated density. Preliminary experiments confirmed that the PCR conditions and the image analysis system were in the linear range of detection for all amplicons. Figure 1 illustrates the linear range of amplification for the COX-1 and COX-2 genes.

Statistical analysis

Data are represented as mean ± SEM. Differences between the control and the different anesthetic drugs were analyzed by Analysis of Variance test. Multiple comparisons were obtained by the Tukey-Kramer method. Statistical significance was accepted at $P < 0.05$.

Results

Figure 1 depicts a representative gel of the COX-1, COX-2, eNOS and iNOS mRNA levels in the lungs of control and anesthetized animals. It can be seen that urethane decreased message for all but eNOS.

Following urethane anesthesia, COX-1 and COX-2 mRNA levels in the lungs were significantly reduced ($P < 0.01$) to 30% of the control levels (Figs. 2 and 3). No significant change in the mRNA levels was observed with the other two anesthetic drugs (Fig. 3).

No significant change in eNOS mRNA levels was observed, but urethane completely suppressed iNOS expression in the lung (Fig. 4). The lung tissue β-Actin/GAPDH ratios were similar for all anesthesia groups, indicating that the observed COX and iNOS mRNA level changes with urethane were likely related to the expression of the enzymes and not the GAPDH housekeeping gene (data not shown).

Table 1. Primer sequences, size, number of PCR cycles and relevant references.

Primer	Upstream	Downstream	Size/Number of PCR Cycles
GAPDH (25)	5′-CGG AGT CAA CGG ATT TGG TCG TAT- 3′	5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′	306/22
β-Actin (25)	5′-CGT GGG CCG CCC TAG GCA CCA-3′	5′-TTG GCC TTA GGG TTC AGG GGG G-3′	243/19
COX-1 (26)	5′-CCT TCT CCA ACG TGA GCT ACT A-3′	5′-GTG GAG AAG AGC ATC AGA CC-3′	486/27
COX-2 (26)	5′-CCT TCC TCC TGT GGC TGA TG-3′	5′-GGA ACA GTC GCT CGT CAT CC-3′	536/29
ENOS (27)	5′-GTG GAC ACA AGG CTG GAG GA-3′	5′-TCC AGT GTC CAG ACG CAC CA-3′	360/29
INOS (28)	5′-ACA ACA GGA ACC TAC CAG CTC A- 3′	5′-GAT GTT GTA GCG CTG TGT GTC A-3′	629/29

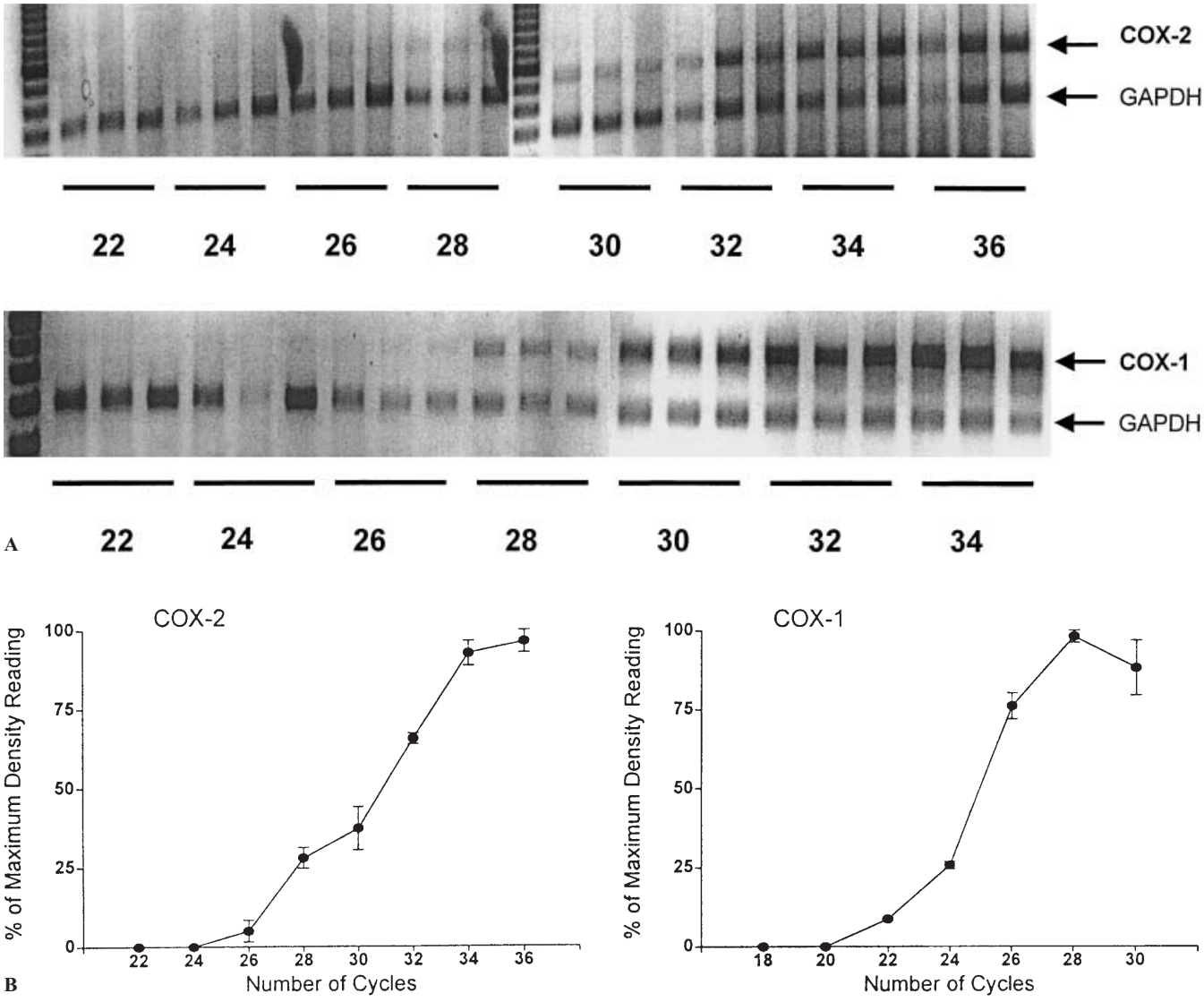

Fig. 1. Panel A: COX-2 and COX-1 amplicons at different cycle numbers to validate the linearity of amplification. Panel B: COX-2/GAPDH or COX-1/GAPDH% of maximum integrated optical density according to the number of PCR cycles.

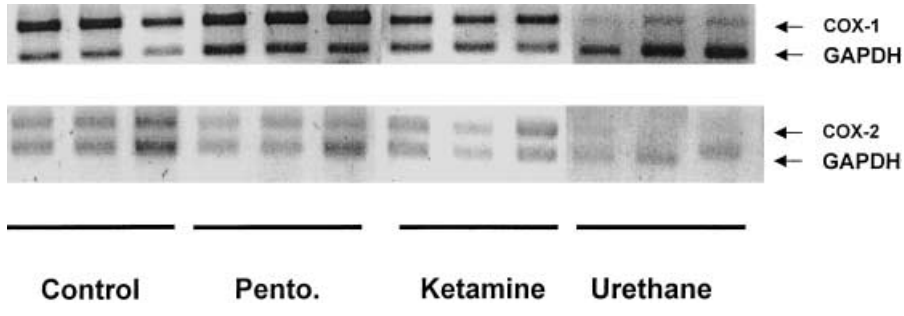

Fig. 2. Representative gel of COX-1, COX-2 expression as determined by RTPCR in rat lung tissue following Pentobarbital (Pento.), Ketamine, Urethane and no anesthesia (Control). Gels containing ethidium bromide were photographed under UV light.

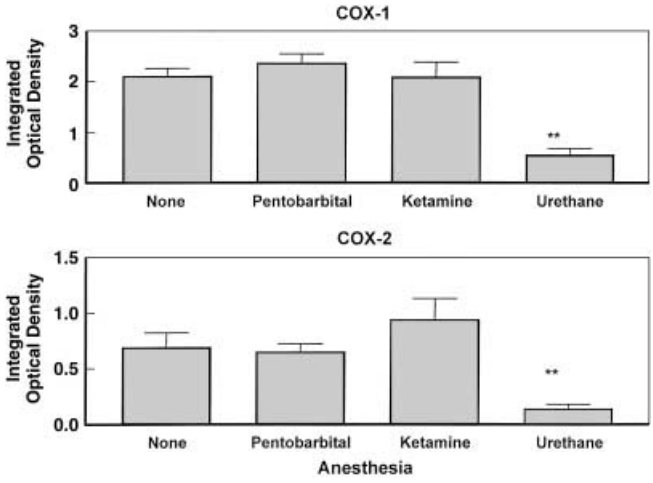

Fig. 3. Lung tissue COX-1 and COX-2 mRNA levels normalized for GAPDH for the different types of anesthesia utilized and control tissues (none). ** $P < 0.01$ as compared to control group.

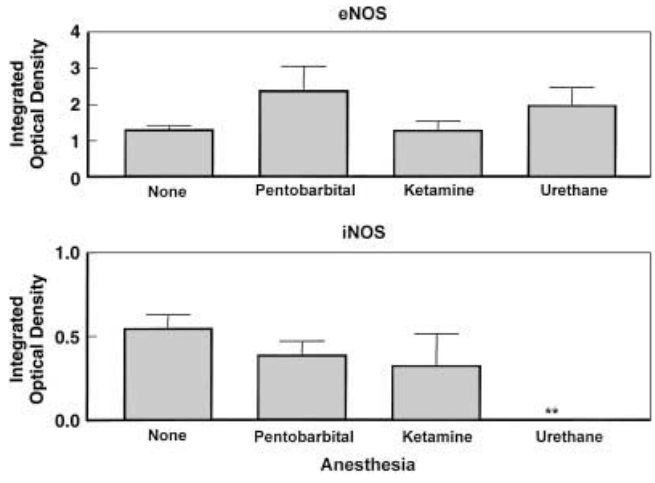

Fig. 4. Lung tissue eNOS and iNOS mRNA levels normalized for GAPDH for the different types of anesthesia utilized and control tissues (none). ** $P < 0.01$ as compared to control group.

Discussion

In the present study we evaluated the effect of urethane upon the basal expression of cyclooxygenases and nitric oxide synthases, hypothesizing that this agent has the potential to modulate mRNA levels for these important enzymes. A significant reduction in the basal COX-1, COX-2 and iNOS mRNA levels in lung tissue following urethane anesthesia has been documented in this study. Such changes were not observed following pentobarbital sodium or ketamine-xylazine anesthesia suggesting that these phenomena were not due to the anesthetized state, but urethane itself led to the decrease in the mRNA levels.

Research dating back as far as 30 years ago produced data to suggest that urethane in rodents has a marked anti-inflammatory and immunosuppressive effect. In fact at least one published report addressed the issue of discovering N-substituted urethane compounds that could be clinically used as anti-inflammatory agents [15].

The mechanism responsible for the anti-inflammatory effects of urethane is unclear. Previous investigators suggested that it could be the result of the drug-induced epinephrine and cortisone levels increase [10]. Recent evidence indicates that following endotoxemia in rats, urethane suppresses TNF-α, a known mediator of the inflammatory response [9].

The effects of urethane upon the cyclooxygenases and nitric oxide synthases have not been previously evaluated. Yet there is evidence to suggest that this drug may affect the expression and/or activity of these enzymes. Cao et al. [16], studying the involvement of COX-2 in LPS induced fever, observed that urethane anesthesia was associated with a significant reduction in COX-2 mRNA in telencephalic neurons. The authors interpreted the reduction in basal COX-2 mRNA expression in the neurons as being related to the decreased neuronal activity provided by the anesthetic.

In the present study we documented that urethane, in contrast to pentobarbital sodium and ketamine-xylazine when utilized at doses to induce surgical depth anesthesia decreases COX-1 and COX-2 lung mRNA levels. This suggests that in the lung urethane, and not the anesthetic state, is responsible for the modulation of cyclooxygenases message levels.

There is also evidence that urethane affects nitric oxide synthesis in rats [17]. A chemical compound closely related to urethane, N-nitroso-N-methyl urethane (NNMU) induces lung injury apparently by a mechanism that involves the nitric oxide pathway [18, 19]. In this study we showed that urethane, as opposed to the other two utilized anesthetics, does modulate the iNOS mRNA levels corroborating with the above findings. The observed reduction in iNOS, but not eNOS mRNA levels following urethane exposure is in keeping with its anti-inflammatory properties. The inducible synthase is the isoform known to be involved in inflammation [12].

The observation that urethane may have an anti-inflammatory action via modulation of message, as opposed to inhibition of the cyclooxygenase and nitric oxide synthase enzymes, is of interest. Non-steroidal anti-inflammatory agents have been reported to inhibit the activity of these enzymes and also suppress the inducible isoform message levels in other organs and cell culture preparations [20–24]. In the rat lung, our data indicate that urethane not only suppresses the inducible COX and NOS isoforms, but also the constitutive COX-1 mRNA expression.

In summary, we have documented a significant reduction in the basal constitutive and inducible cyclooxygenases and inducible nitric oxide synthase mRNA levels in the rat lung. Although urethane and its metabolites have not been found to be of clinical use, the observed suppressive effect of urethane on the cyclooxygenase system makes it an undesirable anesthetic agent when evaluating processes that may involve the inflammatory pathways in the rat lung.

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