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Cloning and Characterization of Cyclooxygenase-1b (putative COX-3) in Rat

James A. Snipes, Bela Kis, Gregory S. Shelness, James A. Hewett, David W. Busija

Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA (J.A.S., B.K., D.W.B.)

Department of Pathology, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA (G.S.S.)

Department of Neuroscience, University of Connecticut Health Center, Farmington, CT 06030, USA (J.A.H.)

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Corresponding Author: Bela Kis, MD, PhD

Department of Physiology and Pharmacology

Wake Forest University Health Sciences

Medical Center Boulevard, Winston-Salem, NC-27157, USA

Email: bkis@wfubmc.edu

Tel.: 1-336-716-4367

Fax: 1-336-716-0504

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Abbreviations:

COX: cyclooxygenase

CEC: cerebral endothelial cell

DMEM: Dulbecco's Modified Eagle's Medium

EBSS: Earl's balanced salt solution

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Abstract

A splice variant of cyclooxygenase-1 (COX-1), COX-1b (previously was termed as COX-3), has been identified in canine tissues as an acetaminophen-sensitive isoform, but the sequence of COX-1b mRNA and the encoded protein are not known in rats. We cloned and sequenced rat COX-1b mRNA from cerebral endothelial cells. Sequence analysis indicated that the 98 bp intron 1 of COX-1 gene remains unprocessed in the COX-1b mRNA causing a frameshift mutation and a 127 amino acid open reading frame with no sequence similarity with known cyclooxygenases. Transient and permanent transfection of COS-7 cells with a vector containing the rat COX-1b cDNA resulted in synthesis of a protein of the expected size. We generated an affinity-purified polyclonal antibody against the rat COX-1b protein. Western blot analysis of rat tissues using this antibody demonstrated the likely existence of rat COX-1b protein *in vivo* with the highest expression in heart, kidney and neuronal tissues. Our results on both stable and on transiently transfected COS-7 cells suggest that rat COX-1b does not have cyclooxygenase activity and does not have any effect on the inhibition of prostaglandin production by acetaminophen. Because this protein has a completely different amino acid sequence than COX-1 and COX-2 and it does not have cyclooxygenase activity we suggest a name **C**yclo**O**xygenase **VA**riant **P**rotein (COVAP) to distinguish it from the known prostaglandin synthesizing cyclooxygenase isoforms.

A new member of the cyclooxygenase family, cyclooxygenase-3 (COX-3), has been identified and characterized in canine tissues (Chandrasekharan et al., 2002) and it has been suggested as the long sought target of acetaminophen (Chandrasekharan et al., 2002). Canine COX-3 mRNA is identical to the COX-1 mRNA except that the intron 1 is retained. Since the normal start codon resides in exon 1 and the 90 base pair (bp) intron 1 sequence maintains the open reading, canine COX-3 mRNA creates an enzymatically active COX-1-related peptide containing a 30 amino acid insertion near the N-terminus (Chandrasekharan et al., 2002). Although different pharmacological characteristics have been reported for canine COX-3 compared to COX-1 or COX-2 (Chandrasekharan et al., 2002), it is a splice variant of COX-1 therefore it should have been named accordingly. We and others (Davies et al., 2004) think that the name COX-3 should be reserved for the product of an independent third cyclooxygenase gene, which has not yet been identified. We prefer to use the term COX-1b instead of COX-3 which better reflects the relations of this cyclooxygenase variants.

Recently, COX-1b mRNA has been detected in tissues from rat (Kis et al., 2003b; Kis et al., 2004a), mouse (Shaftel et al., 2003), and human (Chandrasekharan et al., 2002; Dinchuk et al., 2003). In particular, we have shown that COX-1b mRNA is relatively abundant in cultured cerebral endothelium (Kis et al., 2003b) as well as in freshly harvested cerebral microvessels (Kis et al., 2004a) of rat and that these preparations are unusually sensitive to the inhibition of prostaglandin synthesis by acetaminophen (Kis et al., 2004b). Retention of intron 1, however, which is 98 bp in rat (GeneBank NW_047653.1) and mouse (GeneBank NT_039206.2), and 94 bp in human (GeneBank NT_017568) should lead to a shift in the reading frame and to the synthesis of a protein completely unlike COX-1 and with questionable cyclooxygenase activity. Although not yet tested, it is possible that factors such as a different initiation site related to the insertion of

intron 1 or alternative downstream splicing will restore the reading frame so that a fully functioning cyclooxygenase variant is produced.

This study had five purposes. First, to determine whether the entire intron-1 is inserted into the COX-1b mRNA in rat. We used primary cultures of rat cerebral endothelial cells (CECs) as well as freshly harvested cerebral microvessels because they show abundant expression of COX-1b mRNA (Kis et al., 2003b; Kis et al., 2004a). Second, to determine the complete sequence of the rat COX-1b mRNA and to predict the amino acid sequence of the protein likely to be produced. Third, to transfect COS-7 cells with a rat COX-1b mRNA construct to determine whether it will lead to the synthesis of the predicted protein. Fourth, to generate an antibody against the rat COX-1b mRNA-encoded protein and to demonstrate the existence of this protein *in vivo* in rat tissues. Fifth, to examine the cyclooxygenase activity of the predicted protein and its sensitivity to acetaminophen and also its interaction with COX-2.

We show here that rat COX-1b mRNA retains the entire intron-1 from the parent COX-1 gene. Retention of the 98 bp intron-1 in rat causes a frameshift resulting in a predicted 127 amino acid protein with no COX-1 sequence similarity. Transfection of COS-7 cells with the rat COX-1b cDNA resulted in the detection of a protein of the predicted size. Generation of an antibody against the rat COX-1b mRNA-encoded protein provided evidence for the existence of this protein *in vivo*. Our experiments also demonstrated that the protein encoded by rat COX-1b does not have cyclooxygenase activity and has no influence on the sensitivity of COX-2 to acetaminophen.

Materials and Methods

Culturing rat cerebral endothelial cells

Wistar rats were obtained from Harlan (Indianapolis, IN). All animal experiments were approved by the Animal Care and Use Committee of Wake Forest University Health Sciences. Primary rat CECs were isolated and cultured in collagen type IV and fibronectin coated 35 mm dishes as described previously (Kis et al., 1999). Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 20 % fetal bovine plasma derived serum (Animal Technologies Inc., Tyler, TX), 2 mM glutamine, 1 ng/mL basic fibroblast growth factor, 50 μg/ml endothelial cell growth supplement (BD Biosciences, Bedford, MA), 100 μg/mL heparin, 5 μg/mL vitamin C, and antibiotics. Confluent cultures (4-5th day *in vitro)* consisted of more than 95 % of rat CECs verified by positive immunohistochemistry for von Willebrand factor, and negative immunochemistry for glial fibrillary acidic protein and α-smooth muscle actin.

Isolation of rat cerebral microvessels

Halothane anesthetized male Wistar rats (body weight 200±20 g) were transcardially perfused with chilled saline containing 1000 U/l heparin. Cerebral cortices were freed from larger vessels, pial membranes, and myelin; then were finely minced and incubated in DMEM containing collagenase (200 U/ml, Worthington, Lakewood, NJ) and DNase (30 U/ml, Sigma, St. Louis, MO, USA) at 37 °C for 2 h. After incubation the digested tissue was triturated, mixed with 20% bovine serum albumin in DMEM, and centrifuged at 1000 *g* for 20 min. The pellet containing the microvessels was washed twice with phosphate-buffered saline and stored on dry ice until total

RNA isolation. After further processing this preparation leads to viable CECs that can be used for culturing (Kis et al., 1999).

RNA isolations and RT-PCR

Total RNA was isolated from the samples by SV Total RNA Isolation System (Promega, Madison, WI). The poly(A) mRNA fraction was isolated by PolyATract mRNA Isolation System (Promega). RT-PCR experiments were carried out as described previously (Kis et al., 2003b). The design of the specific primers for rat COX-1b detection was based on the hypothesis that the rat COX-1b mRNA is identical to the full length form of COX-1 except that it retains intron 1, as in the dog. The sense primer (5'-CAGAGTCATGAGTCGTGAG; 1376773-1376791 bp of GeneBank NW_047653.1) was designed to bind to the 5' end of the putative rat COX-1b including the start codon of the COX-1 gene and part of intron 1. The antisense primer (5'-AGAGGGCAGAATGCGAGTAT; 501-520 bp of GeneBank S67721) binds to exon 5 of the rat COX-1 gene. The expected length of the RT-PCR product was 573 base pairs. Our COX-1b primer set distinguishes between COX-1 and COX-1b, and it also distinguishes between COX-1b and "partial" cyclooxygenases (PCOX-1a, PCOX-1b) because our antisense primer binds to exon 5 which is lacking in PCOXs (Chandrasekharan et al., 2002).

Cloning and sequencing of the rat COX-1b mRNA

Amplified RT-PCR product was separated on a 3% NuSieve agarose gel (Cambrex, Rockland, ME) and purified using Wizard SV gel clean up system (Promega). To amplify and accurately sequence both ends of the COX-1b fragment, the purified RT-PCR product was ligated into pGEM-T Easy Vector (Promega) and transformed into *E. coli* (JM109, Promega). Automated

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DNA sequencing of the COX-1b construct was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Expression of the protein encoded by rat COX-1b mRNA in COS-7 cells

To examine whether the COX-1b mRNA expresses the predicted protein, we amplified the sequence of the COX-1b inserted into the pGEM-T Easy Vector. The sense primer (5'-TAATACGACTCACTATAGGG) was designed to bind to the T7 promoter. The antisense primer (5'-CGGTCTAGAGCAGGAAAT) was designed to bind to the 3' end of the COX-1b sequence. The 3' primer lacked the COX-1b stop codon and contained an *Xba I* restriction site for creation of a C-terminal 3x FLAG tagged form of COX-1b. For this purpose, the PCR product was digested with *Not I* and *Xba I* and inserted into *Not I*-*Xba I*-digested p3XFLAG-CMV-14 expression vector (Sigma). For transient transfection COS-7 cells were cultured in 24-well plates and were transfected with the COX-1b-FLAG construct (0.5 μg plasmid/well) by TransFast transfection reagent (Promega). Twenty-four hours after transfection, proteins were isolated and subjected to Western blot analysis using antibody against the FLAG epitope. COS-7 cells transfected with p3XFLAG-CMV-7-BAP plasmid were used as a positive control. For stable transfection COS-7 cells were cultured in 60 mm dish and were transfected with the COX-1b–FLAG (5 μg plasmid/dish) by TransFast transfection reagent (Promega). One day after transfection the culture medium was supplemented with 500 μg/ml geneticin (G418; Gibco). Surviving colonies were trypsinized in cloning rings and transferred to 12-well plates for further propagation in the presence of the selective medium. Proteins were isolated from the third generations of clones of stable transfected cells and were subjected to Western blot analysis using anti-FLAG monoclonal antibody.

Production of COX-1b protein in *E. coli*

Primers (sense primer: 5'-CCCCATATGAGTCGTGAGGTATAC, antisense primer: 5'-ACAGGATCCTGTCAGCAGGA) containing *NdeI* and *BamHI* restriction sites were used to amplify rat COX-1b cDNA. The PCR product was inserted into the *NdeI* and *BamHI* restriction sites of the pET-14b expression vector (Novagen, Madison, WI) and transformed into BL21(DE3)pLysS competent *E. coli* (Promega). Cells were harvested and His-tagged COX-1b protein was extracted using Ni-NTA His-Bind Purification Kit (Novagen) according to the manufacturer's instructions. This purified protein was used as a positive control in Western blots using our anti-rat COX-1b antibody (see below).

Co-transfection of COS-7 cells with COX-2 and COX-1b

The rat COX-2 cDNA in pcDNA3.1 vector was constructed as previously described (Vidwans et al., 2001). The rat COX-1b expression vector was created by digesting the pGEM-T Easy Vector containing the rat COX-1b with *NotI* and *SpeI* and the purified product was inserted using *NotI* and *XbaI* restriction sites into pcDNA3.1 vector. The resulting plasmid DNAs (pcDNA3.1-COX-2 and pcDNA3.1-COX-1b) were amplified in *E. coli* and purified for transfection with a DNA purification system (Promega). COS-7 in 60 mm dishes were transfected at ~50% confluence with empty pcDNA3.1 plasmid (10 μg/dish), pcDNA3.1-COX-2 plus empty pcDNA3.1 plasmids (5 μg/dish from each), pcDNA3.1-COX-1b plus empty pcDNA3.1 plasmids (5 μg/dish from each), or with pcDNA3.1-COX-2 plus pcDNA3.1-COX-1b plasmids (5 μg/dish from each) using TransFast transfection reagent (Promega). One day after the transfection the cells

were split into 12-well plates for further experiments. The expression of COX-2 and COX-1b proteins was assessed by Western blot analysis.

Production of polyclonal anti-COX-1b antibodies

Peptides corresponding to the N-terminal 2-17 amino acids of rat COX-1b mRNA encoded protein (SRESDPSGAPTRPGIR), as predicted by cDNA sequences, were synthesized and coupled to keyhole limpet hemocyanin (KLH). The peptide-KLH complexes were injected into New Zealand White rabbits 4 times at three week intervals. The serum of the rabbit was collected 77 days after the first injection. The resulting polyclonal antibodies were then affinity purified using the above peptide immobilized on Affi-Gel column (Bio-Rad, Hercules, CA).

Subcellular fractionation

Confluent cultures of rat CECs were washed two times with phosphate buffered saline. Then the cells were scraped from tissue culture dishes and collected in microcentrifuge tubes. Cells were recovered by centrifugation for 5 min at 1000 g, were resuspended in ice-cold hypotonic buffer (10 mM HEPES, pH 7.4, 1 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, 1μg/ml leupeptin), incubated on ice for 15 min, and then were homogenized with a 2 ml Dounce homogenizer. The homogenate was adjusted to 250 mM sucrose using a 2 M stock and centrifuged at 700 g for 10 min. The post-nuclear supernatant was transferred into a fresh microcentrifuge tube and subjected to an additional round of centrifugation at 700 g to ensure complete removal of nuclei. The supernatant from the second 700 g spin was then centrifuged at 120,000 g for 1 hr using a Beckman TL-100 ultracentrifuge (Beckman Instruments, Fullerton, CA).

The resulting pellet consisted of the total post-nuclear membrane fraction and the supernatant contained the cytosol.

Western blotting

Proteins were isolated from samples using NP40 lysis buffer supplemented with proteinase inhibitors (1 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride, 1μg/ml leupeptin) as described previously (Kis et al., 2003a). Western blot membranes were prepared (Kis et al., 2003a) and the blots were incubated with monoclonal anti-FLAG (1:2000; Sigma), polyclonal anti-COX-2 (1:5000; Cayman Chemical, Ann Arbor, MI) or polyclonal anti-COX-1b antibodies (1:2000) overnight at 4 °C. The membranes were then washed three times in Tris buffered saline with 0.1 % Tween 20 and then incubated for 1hr in the blocking buffer with anti-rabbit IgG (1:50000; Jackson Immuno Research, West Grove, PA) or anti-mouse IgG (1:5000; Jackson Immuno Research) conjugated to horseradish peroxidase. The final reaction products were visualized using enhanced chemiluminescence (SuperSignal West Pico; Pierce, Rockford, IL) and recorded on x-ray film.

Immunocytochemistry

Primary cultures of rat CECs were cultured on glass coverslips. Cultures were washed with phosphate buffered saline, fixed in 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. The rat CECs were incubated with polyclonal rabbit anti-rat COX-1b primary antibody (1:200) at room temperature for 60 min. and then incubated with FITC-labeled anti-rabbit (1:100; Vector Labs, Burlingame, CA) secondary antibody at room temperature for 30 min. Confocal images of cellular fluorescence were acquired on a Zeiss LSM 510 laser scanning microscope (Zeiss, Jena, Germany).

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Prostaglandin E_2 (PGE_2) measurement from transfected cells

COX-1b p3XFLAG-CMV-14 stable transfected COS-7 cells were cultured in 12-well plates. Confluent cultures were washed twice with 37^{o}C DMEM and then the cells were incubated at 37^{o}C for 15 min in DMEM containing arachidonic acid (30 μM) with or without acetaminophen (100 μM). After the incubation, media were collected and stored at -60 ^{o}C until assayed.

COX-2 and/or COX-1b transiently transfected COS-7 cells were cultured in 12-well plates. Confluent cultures were washed twice with 37^{o}C Earl's balanced salt solution (EBSS; Na^+: 143.6 mM, K^+: 5.4 mM, Ca^{2+}: 1.8 mM, Mg^{2+}: 0.8 mM, Cl^-: 125.3 mM, HCO_3^-: 26.2 mM, $H_2PO_4^-$: 1.1 mM, SO_4^{2-}:0.8 mM, glucose: 5.5 mM). The cells were then incubated for 30 min in EBSS containing 15 μM arachidonic acid, L-glutamine (2 mM) with or without acetaminophen. After the incubation, media were collected and stored at -60 ^{o}C until assayed. PGE_2 concentrations in media were measured with a specific ELISA (Oxford Biomedical Research, Oxford, MI).

Results

Using mRNA from cultured rat CECs and isolated brain microvessels, a COX-1b PCR product of 573 bp was generated. The sequence showed 100% identity with the respective exonic parts and the entire 98 bp long intron-1 of the rat COX-1 gene located on chromosome 3 (Fig. 1). Sequence analysis revealed that the rat COX-1b mRNA encodes a predicted 127 amino acid protein with a molecular weight of approximately 13 kDa (Fig. 1). COX-1b protein did not show homology to any existing database entries. The mRNA sequences of rat COX-1b isolated from CECs and from cerebral capillaries have been deposited in the GenBank with accession numbers AY523672 and AY523673, respectively. RT-PCR experiments demonstrated the presence of the COX-1b sequence in the poly(A) RNA fraction isolated from CECs suggesting that the COX-1b sequence is polyadenylated which is a necessary step during the formation of mature mRNA (Fig. 2).

When we transiently transfected COS-7 cells with the p3XFLAG-CMV-14 vector containing the cDNA of COX-1b we detected a clear protein band by Western blot analysis using the anti-FLAG antibody. The COX-1b/FLAG fusion protein was detected at a molecular weight of about 17 kDa (Fig. 3A) which compares favorably with the calculated molecular weight of 16.6 kDa for the COX-1b/FLAG fusion protein. As a positive control, COS-7 cells were transfected with the p3XFLAG-CMV-7-BAP plasmid and they showed the expression of the bacterial alkaline phosphatase/FLAG fusion protein (calculated molecular weight 69 kDa). We did not detect specific bands in untransfected cells (Fig. 3A).

During our attempt to establish clones of COS-7 cells stably transfected with COX-1b p3XFLAG-CMV-14 vector, we successfully subcultured five geneticin resistant colonies. Western

blot analysis using anti-FLAG antibody detected bands of COX-1b/FLAG fusion protein in three of the five stable transfected clones (Fig. 3B).

To demonstrate the existence of COX-1b protein in rat tissues we generated affinity-purified polyclonal antibodies against the rat COX-1b protein. The antibody recognized the purified COX-1b protein produced in *E. coli* (Fig. 4, 5). In addition to the COX-1b protein other bands were also visualized in our Western blots. These additional bands are easily distinguished from COX-1b and most of them also appeared when incubation with the primary antibody was omitted (Fig. 5). However, in neuronal tissue samples there is a strong band at ~60 kDa which is related to the primary antibody (Fig. 4, 5). This protein, which is recognized by the anti-COX-1b antibody seems to be expressed primarily in neurons (Fig. 6).

We checked the expression of COX-1b protein in twelve different rat tissue samples. COX-1b was expressed in all tissues we examined; the expression was the strongest in the heart, kidney and neuronal tissues (Fig. 4, 5). Following subcellular fractionation of cultured rat CECs we observed COX-1b protein predominantly in the cytosolic fraction, with weak staining in the nuclear fraction and no detectable signal in post-nuclear membranes (Fig. 7A). Our immunocytochemistry experiments on primary cultures of rat CECs showed a slightly punctate staining pattern distributed throughout the cytoplasm (Fig. 7B). When the incubation with the primary anti-COX-1b antibody was omitted during the immunocytochemistry, no staining was visible.

To determine the cyclooxygenase activity of the COX-1b protein, first we used COS-7 cells which were stable transfected with the COX-1b/FLAG fusion protein. We measured the PGE_2 production in COX-1b positive (clone 2 on Fig. 3B) and COX-1b negative (clone 3 on Fig. 3B) stably transfected COS-7 cells in the presence of exogenous arachidonic acid. We could not

detect a significant difference between the PGE_2 production in COX-1b positive and COX-1b negative COS-7 cells (Fig. 8). Acetaminophen did not have a significant effect on the PGE_2 production in either cell line (Fig. 8). In a second series of experiments we transiently transfected COS-7 cells with native COX-1b (without FLAG epitope), with murine COX-2, and co-transfection with both COX-2 and COX-1b. Western blot analysis demonstrated the expression of the respective proteins in the transfected cells (Fig. 9A). The PGE_2 production of COX-1b transfected cells was not different compared to the empty vector transfected control cells (Fig. 9B). COX-2 transfection resulted in a three-fold increase in PGE_2 production, which was slightly inhibited by low a concentration of acetaminophen. The COX-2/COX-1b co-transfected cells showed similar PGE_2 production profile as the COX-2 only transfected cells (Fig. 9B).

Discussion

There are five major findings of our present study. [1] This is the first study which describes the entire sequence of the rat COX-1b mRNA. [2] We demonstrated that the rat COX-1b mRNA encodes a protein, which has a completely different amino acid sequence than the known cyclooxygenases. [3] We generated an antibody against the rat COX-1b protein which is suitable for Western blot analysis. [4] We demonstrated that COX-1b protein probably exists *in vivo* in multiple rat tissues. [5] COX-1b apparently does not have cyclooxygenase activity.

COX-1b (originally named as COX-3) was identified in canine tissues as the long sought brain specific cyclooxygenase enzyme which is the target of acetaminophen (Chandrasekharan et al., 2002), thus potentially solving the mystery of the mechanism of action of this drug (Botting, 2000). Although COX-1b as a COX-1 splice variant was demonstrated in rats, mice and humans, there was a serious question as to whether COX-1b mRNA in these species also encode an acetaminophen-sensitive cyclooxygenase as in canines, because in these species the retention of the entire intron-1, which is 98 bp in rat and mouse and 94 bp in human, would shift the original reading frame of COX-1 (Dinchuk et al., 2003; Schwab et al., 2003). Hypothetically a different initiation site or an alternative downstream splicing might restore the original COX-1 reading frame resulting in the synthesis of a peptide with homology to COX-1, except the N-terminal end which would be encoded by the inserted intron-1. To address this hypothesis it was necessary to clone and sequence the entire rat COX-1b mRNA. We cloned and sequenced a 573 bp long COX-1b mRNA fragment which showed that the entire 98 bp long intron-1 is inserted into the rat COX-1b mRNA (Fig. 1). We considered all three reading frame possibilities based upon the sequence of the detected 573 bp long COX-1b mRNA fragment. First, maintaining the original start codon for COX-1 mRNA defines an open reading frame with a stop codon at base 382 (Fig. 1). Because the

available sequence of the 5' end is very limited, we cannot rule out the possibility of a more proximal translation initiation site for the COX-1b mRNA. The other two possible reading frames, however, indicate premature stop codons at bp 2 or 9 (Fig. 1). Therefore, even if present, a more proximal translation initiation site than known for COX-1 could not save the original COX-1 reading frame.

How the COX-1 variant mRNA transcript avoids the nonsense-mediated decay pathway, which specifically targets mRNAs containing premature stop codons for degradation (Hentze and Kulozik, 1999; Lynch and Kewalramani, 2003), is currently not known. A mRNA is targeted by nonsense-mediated decay by the presence of a *cis*-acting downstream element appropriately located within 200 nucleotides downstream of the stop codon (Ruiz-Echevarria and Peltz, 1996; Ruiz-Echevarria et al., 1998). Conversely, mRNA containing premature stop codons can avoid nonsense-mediated decay if they possess a *cis*-acting stabilizer element (Ruiz-Echevarria et al., 2001). Thus, it is possible that the COX-1 variant mRNA lacks a properly positioned downstream element or carries a stabilizer element.

We detected the COX-1b sequence with RT-PCR in poly (A) RNA samples isolated from rat CECs, which suggested that the COX-1b sequence may exist in a form of mature mRNA which makes it highly possible that this sequence is translated into a protein. According to our sequence analysis, the only possible protein which could be translated from the rat COX-1b mRNA is a 127 amino acid protein which has a completely different amino acid sequence than the known cyclooxygenases. Initially we did not have an antibody against this hypothetical COX-1b protein and therefore we inserted the rat COX-1b cDNA into the p3XFLAG-CMV-14 vector proximal to the sequence of the FLAG protein. When we transfected COS-7 cells with this construct we detected the COX-1b/FLAG fusion protein with anti-FLAG antibody in Western blot analysis at

the expected molecular weight. This was the first evidence that the rat COX-1b mRNA contains an open reading frame and can be translated into a protein. We have also generated clones of COS-7 cells which were stable transfected with the COX-1b p3XFLAG-CMV-14 vector. Western blot analysis demonstrated the synthesis of the COX-1b/FLAG fusion protein in these stable transfected COS-7 cells.

To explore the possible expression of COX-1b in rat tissues samples we generated an anti-rat COX-1b antibody. Western blot analysis demonstrated that the antibody specifically binds to the purified rat COX-1b protein produced in *E. coli* (Fig. 5). We tested the antibody further on protein samples isolated from COS-7 cells which were transfected with the COX-1b p3XFLAG-CMV-14 vector (Fig. 3C). This experiment also proved that our anti-COX-1b antibody was specific and sensitive enough to be used in Western blot analysis. Using this antibody we detected COX-1b protein in 12 different rat tissue samples demonstrating that COX-1b protein may be synthesized and may exist *in vivo*. The expression was strongest in the heart, kidney and neuronal tissues (Fig. 4, 5). Among the neuronal tissues it seems that the COX-1b protein expression is the highest in spinal cord which correlates well with our previous RT-PCR data which demonstrated the highest COX-1b mRNA expression in the spinal cord among the neuronal tissues we studied (Kis et al., 2004a). Besides the band of the COX-1b protein some other bands were also visualized in our Western blots. These additional bands are easily distinguished from the band of COX-1b according to their molecular weight and most of them are a result of non-specific binding of the anti-rabbit secondary antibody to rat proteins (Fig. 4, 5). The non-specific binding was epecially strong in the spleen samples, presumably due to the high immunocompetent cells/antibody content of the spleen. In neuronal tissue samples an additional protein was recognized by the rat anti-COX-1b antibody. Our search of protein databases revealed that the hapten peptide, which was

used to induce antibody production (amino acids 2-17 of the rat COX-1b), shows homology to the sequence of an unnamed protein with a molecular weight of 64 kDa (gi: 34857947). Therefore our polyclonal antibody might also bind to this 64 kDa protein which would be at the right location on the blot to account for this additional band. This protein is highly abundant in cultured rat cortical neurons (Fig. 6) which can explain why we detected the strong additional band in neuronal tissues by Western blot analysis.

COX-1b does not show homology to any protein sequences of published databases. It has a very basic character with the estimated pI of 12.40. In addition to the abundance of basic amino acids, the protein is also quite proline rich (11.81% proline) arguing against the formation of extensive alpha-helical domains and for the possible formation of beta turns and perhaps the formation of antiparallel beta-sheets. Hyrophobicity analysis by the method of Kyte and Doolittle (Kyte and Doolittle, 1982) did not reveal the existence of transmembrane domains. However, the protein displayed an unusual periodicity, perhaps giving rise to an amphipathic character responsible for peripheral association with intracellular membrane surfaces. This may explain COX-1b's punctate distribution in CECs. Other motifs including a possible protein kinase C phophorylation site and cysteine rich domains were also noted in the amino acid sequence. The analysis did not identify a signal peptide in the molecule suggesting that COX-1b is not a secretory protein.

Subcellular fractionation of cultured CECs (Fig. 7A) and rat tissues (data not shown) followed by Western blot analysis suggests that COX-1b is a cytosolic protein because it was detected predominantly in the cytosolic fraction of the preparations. Immunostaining of primary cultures of rat CECs confirmed this result showing that COX-1b immunoreactivity is not localized

to specific intracellular structures, rather it appeared as a punctate pattern distributed throughout the cytoplasm (Fig. 7B).

The rat COX-1b has an amino acid sequence completely different from COX-1 or COX-2 which makes it unlikely that COX-1b is involved in prostaglandin production. We used stable and transiently transfected COS-7 cells to determine the cyclooxygenase activity of COX-1b. COS-7 cells were chosen specifically, because Vidwans et al. (Vidwans et al., 2001) showed that these cells have little endogenous cyclooxygenase activity, but when they were transfected with COX-2 construct their PGE_2 production increased several fold. Our current findings support these results. Our results on both stable and transiently transfected cells suggest that rat COX-1b does not have cyclooxygenase activity. Moreover COX-1b did not have any effect on the inhibition of COX-2 by acetaminophen. To support our findingsWarner *et al.* (Warner et al., 2004) demonstrated in a variety of rat tissues that the production of prostanoids is dependent on the two known isoforms of cyclooxygenase, COX-1 and COX-2, and that there is no evidence for the involvement of a particular acetaminophen-sensitive COX-1b (named COX-3) isoform. On the other hand, our results make it unlikely that the acetaminophen-iduced hypothermia is the consequence of the inhibition of COX-1b as suggested by Ayoub et *al* (Ayoub et al., 2004).

We have also sequenced the mouse COX-1b mRNA (GenBank AY547265) which shows 100% homology to its rat counterpart suggesting that the mouse COX-1b mRNA encoded protein has the same amino acid sequence as in the rat. However, if the whole 94 bp intron-1 is retained in the human COX-1b it will lead to the synthesis of a completely different protein with no similarity to the rat COX-1b.

Our experiments have led us to the discovery of a new protein which is transcribed from COX-1b mRNA, an alternative splice variant of the COX-1 gene. Because this protein has a

completely different amino acid sequence than COX-1 and COX-2 and it does not have cyclooxygenase activity we suggest the name **C**yclo**O**xygenase **VA**riant **P**rotein (COVAP) to distinguish it from the known prostaglandin synthesizing cyclooxygenase isoforms.

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References

Ayoub SS, Botting RM, Goorha S, Colville-Nash PR, Willoughby DA and Ballou LR (2004) Acetaminophen-induced hypothermia in mice is mediated by a prostaglandin endoperoxide synthase 1 gene-derived protein. *Proc Natl Acad Sci U S A* **101**:11165-11169.

Botting RM (2000) Mechanism of action of acetaminophen: is there a cyclooxygenase 3? *Clin Infect Dis* **31 Suppl 5**:S202-210.

Chandrasekharan NV, Dai H, Roos KL, Evanson NK, Tomsik J, Elton TS and Simmons DL (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc Natl Acad Sci U S A* **99**:13926-13931.

Davies NM, Good RL, Roupe KA and Yanez JA (2004) Cyclooxygenase-3: axiom, dogma, anomaly, enigma or splice error?--Not as easy as 1, 2, 3. *J Pharm Pharm Sci* **7**:217-226.

Dinchuk JE, Liu RQ and Trzaskos JM (2003) COX-3: in the wrong frame in mind. *Immunol Lett* **86**:121.

Hentze MW and Kulozik AE (1999) A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* **96**:307-310.

Kis B, Rajapakse NC, Snipes JA, Nagy K, Horiguchi T and Busija DW (2003a) Diazoxide induces delayed pre-conditioning in cultured rat cortical neurons. *J Neurochem* **87**:969-980.

Kis B, Snipes A, Bari F and Busija DW (2004a) Regional distribution of cyclooxygenase-3 mRNA in the rat central nervous system. *Brain Res Mol Brain Res* **126**:78-80.

Kis B, Snipes JA and Busija DW (2004b) Acetaminophen-sensitive prostaglandin production in rat cerebral endothelial cells. *Faseb J* **18**:A676.

Kis B, Snipes JA, Isse T, Nagy K and Busija DW (2003b) Putative cyclooxygenase-3 expression in rat brain cells. *J Cereb Blood Flow Metab* **23**:1287-1292.

Kis B, Szabo CA, Pataricza J, Krizbai IA, Mezei Z, Gecse A, Telegdy G, Papp JG and Deli MA (1999) Vasoactive substances produced by cultured rat brain endothelial cells. *Eur J Pharmacol* **368**:35-42.

Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**:105-132.

Lynch M and Kewalramani A (2003) Messenger RNA surveillance and the evolutionary proliferation of introns. *Mol Biol Evol* **20**:563-571.

Ruiz-Echevarria MJ, Gonzalez CI and Peltz SW (1998) Identifying the right stop: determining how the surveillance complex recognizes and degrades an aberrant mRNA. *Embo J* **17**:575-589.

Ruiz-Echevarria MJ, Munshi R, Tomback J, Kinzy TG and Peltz SW (2001) Characterization of a general stabilizer element that blocks deadenylation-dependent mRNA decay. *J Biol Chem* **276**:30995-31003.

Ruiz-Echevarria MJ and Peltz SW (1996) Utilizing the GCN4 leader region to investigate the role of the sequence determinants in nonsense-mediated mRNA decay. *Embo J* **15**:2810-2819.

Schwab JM, Beiter T, Linder JU, Laufer S, Schulz JE, Meyermann R and Schluesener HJ (2003) COX-3--a virtual pain target in humans? *Faseb J* **17**:2174-2175.

Shaftel SS, Olschowka JA, Hurley SD, Moore AH and O'Banion MK (2003) COX-3: a splice variant of cyclooxygenase-1 in mouse neural tissue and cells. *Brain Res Mol Brain Res* **119**:213-215.

Vidwans AS, Uliasz TF, Hewett JA and Hewett SJ (2001) Differential modulation of prostaglandin H synthase-2 by nitric oxide-related species in intact cells. *Biochemistry* **40**:11533-11542.

Warner TD, Vojnovic I, Giuliano F, Jimenez R, Bishop-Bailey D and Mitchell JA (2004) Cyclooxygenases 1, 2, and 3 and the production of prostaglandin I_2: investigating the activities of acetaminophen and cyclooxygenase-2-selective inhibitors in rat tissues. *J Pharmacol Exp Ther* **310**:642-647.

Footnotes

J.A. Snipes and B. Kis contributed equally to this work.

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Bela Kis, MD, PhD

Department of Physiology and Pharmacology

Wake Forest University Health Sciences

Medical Center Boulevard

Winston-Salem, NC-27157, USA

Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA (J.A.S., B.K., D.W.B.)

Department of Pathology, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA (G.S.S.)

Department of Neuroscience, University of Connecticut Health Center, Farmington, CT 06030, USA (J.A.H.)

Legends to the Figures

Figure 1

Sequence of the rat COX-1 (partial) and COX-1b mRNA and their translation into amino acid sequences

Total RNA was isolated from primary cultures of rat CECs and from rat cerebral microvessels. COX-1b was detected by RT-PCR using specific primers. The PCR product was ligated into pGEM-T Easy Vector and transformed into *E. coli* to amplify and accurately sequence both strands of the rat COX-1b cDNA. Automated DNA sequencing of the COX-1b construct was performed on an ABI Prism 377 DNA sequencer.

Figure 2

Detection of rat COX-1b in poly(A) RNA isolated from rat cerebral endothelial cells

Representative gel electrophoresis of RT-PCR product for COX-1b detected in total RNA and poly(A) RNA fractions isolated from primary cultures of rat CECs. The expected molecular weight of the PCR product was 573 bp. M: molecular weight marker.

Figure 3

COX-1b protein expression in COS-7 cells

A: COS-7 cells were transiently transfected with the COX-1b p3XFLAG-CMV-14 vector containing the cDNA of rat COX-1b (lanes 1-4) or with p3XFLAG-CMV-7-BAP vector (lanes 5-8). Protein expression was demonstrated by Western blot analysis using anti-FLAG antibody. In the lysate of COS-7 cells transfected with the COX-1b p3XFLAG-CMV-14 vector we detected

COVAP/FLAG fusion protein at a molecular weight of about 17 kDa (lanes 1-4). The cells transfected with the p3XFLAG-CMV-7-BAP plasmid expressed the bacterial alkaline phosphatase (BAP)/FLAG fusion protein (lanes 5-8). We could not detect specific bands in untransfected cells (lanes 9-12). The numbers on the left indicate the weights and the positions of the molecular weight markers in kDa.

B: COS-7 cells were transfected with COX-1b p3XFLAG-CMV-14 vector and stable transfected clones were selected by culturing the cells in geneticin containing medium. Protein expression was demonstrated by Western blot analysis using anti-FLAG antibody. This panel shows that three out of the five selected geneticin-resistant clones expressed the COVAP/FLAG fusion protein.

C: Proteins isolated from clones 2 and 3 of the COX-1b p3XFLAG-CMV-14 stable transfected COS-7 cells (see panel B) were subjected to Western blot analysis using anti-COX-1b polyclonal antibody. 2: clone 2; 3: clone 3.

Figure 4

COX-1b expression in rat tissues

Proteins isolated from rat tissues were subjected to Western blot analysis with (A) or without (B) polyclonal anti-rat COX-1b antibody. The numbers on the left indicate the weights and the positions of the molecular weight markers in kDa. 1: heart, 2: liver, 3: lung, 4: aorta, 5: spleen, 6: pancreas, 7: kidney, 8: muscle, 9: brainstem, 10: cerebellum, 11: cortex, 12: spinal cord.

Figure 5

COX-1b expression in rat neural tissues

Proteins isolated from different parts of the rat central nervous system were subjected to Western blot analysis using polyclonal anti-rat COX-1b antibody. Lane 9-11 show the result when incubation with the primary antibody was omitted. S: COX-1b standard produced in *E. coli*, 1,5,9: brainstem, 2,6,10: cerebellum, 3,7,11: cortex, 4,8: spinal cord.

Figure 6

Comparison of the expression of the 64 kDa protein in neurons, cerebral capillaries and cerebral endothelial cells

Proteins isolated from primary cultures of rat CECs, rat cerebral microvessels and primary cultures of rat cortical neurons were subjected to Western blot analysis using polyclonal anti-rat COX-1b antibody.

Figure 7

Intracellular localization of COX-1b protein in cultured rat cerebral endothelial cells

Panel A: Proteins isolated from subcellular fractions of cultured rat CECs and were subjected to Western blot analysis using polyclonal anti-rat COX-1b antibody. 1: nuclear fraction, 2: membrane fraction, 3: cytosolic fraction, S: COX-1b standard produced in *E. coli*. Panel B: immunostaining of primary cultures of rat CECs using anti-rat COX-1b antibody.

Figure 8

PGE_2 production in COX-1b stable transfected COS-7cells

Rat COX-1b expressing (clone 2; see figure 3) and non-expressing (clone 3; see figure 3) stable transfected COS-7 cells cultured in 12-well plates were incubated at 37^{o}C for 15 min in DMEM containing arachidonic acid (30 μM) with or without acetaminophen (100 μM). PGE_2 concentrations in media were measured with ELISA.

Figure 9

PGE_2 production in COX-2 and/or COX-1b transiently transfected COS-7 cells

COS-7 cells were transfected with the empty pcDNA3.1 vector, pcDNA3.1 vector containing rat COX-2 cDNA, pcDNA3.1 vector containing rat COX-1b cDNA or co-transfected with the COX-2 and the COX-1b containing vectors. Panel A shows the expression of COX-2 and COX-1b protein in transfected cells. Panel B shows PGE_2 production in the transfected cells. COS-7 cells cultured in 12-well plates were incubated at 37^{o}C for 30 min in Earl's balanced salt solution containing arachidonic acid (10 μM) with or without acetaminophen (100 μM). PGE_2 concentrations in media were measured by ELISA.

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```
            M  S   ++++++++++++Intron 1++++++++++++++++++++++++++  Cox-1
            M  S  R  E  S  D  P  S  G  A  P  T  R  P  G  I  R  W  Cox-1b
-7   CAGAGTCATGAGTCGTGAGTCCGACCCCAGCGGTGCCCCCACGCGCCCCGGAATTCGGT  52

    ++++++++++++++++++++++++++Intron 1+++++++++++++++++R  R  S   Cox-1
      P  A  G  G  A  L  N  V  R  L  N  S  L  F  P  L  Q  E  G  V  Cox-1b
53  GGCCTGCAGGCGGAGCCTTGAACGTCAGGCTCAACTCTCTCTTCCCTCTGCAGGAAGGAG  112

     L  S  L  Q  F  P  L  L  L  L  L  L  L  L  P  P  P  P  V  L  Cox-1
      S  R  S  S  F  P  C  C  C  S  C  C  C  S  H  H  P  R  Y  C  Cox-1b
113 TCTCTCGCTCCAGTTTCCCCTGCTGCTGCTCCTGCTGCTGCTCCCACCACCCCCGGTACT  172

     L  T  D  A  G  V  P  S  P  V  N  P  C  C  Y  Y  P  C  Q  N  Cox-1
      S  Q  M  L  G  Y  P  H  Q  S  I  P  V  V  T  I  H  A  R  T  Cox-1b
173 GCTCACAGATGCTGGGGTACCCTCACCAGTCAATCCCTGTTGTTACTATCCATGCCAGAA  232

     Q  G  V  C  V  R  F  G  L  D  H  Y  Q  C  D  C  T  R  T  G  Cox-1
      R  V  S  V  S  A  S  A  S  T  T  T  N  V  T  V  L  A  R  A  Cox-1b
233 CCAGGGTGTCTGTGTCCGCTTCGGCCTCGACCACTACCAATGTGACTGTACTCGCACGGG  292

     Y  S  G  P  N  C  T  I  P  E  I  W  T  W  L  R  S  S  L  R  Cox-1
      T  R  A  P  T  V  L  S  L  R  S  G  P  G  F  G  V  P  C  G  Cox-1b
293 CTACTCGGGCCCCAACTGTACTATCCCTGAGATCTGGACCTGGCTTCGGAGTTCCCTGCG  352

     P  S  P  S  F  T  H  F  L  L  T  H  G  Y  W  I  W  E  F  V  Cox-1
      P  A  P  H  S  P  I  S  C  *  ----------------------------  Cox-1b
353 GCCCAGCCCCTCATTCACCCATTTCCTGCTGACACACGGGTACTGGATCTGGGAGTTTGT  412

     N  A  T  F  I  R  E  V  L  M  R  L  V  L  T  V  R  S  N  L  Cox-1
    ------------------------------------------------------------  Cox-1b
413 GAATGCCACCTTCATCCGAGAAGTACTCATGCGCCTGGTACTCACGGTGCGGTCCAACCT  472

     I  P  S  P  P  T  Y  N  T  A  H  D  Y  I  S  W  E  S  F  S  Cox-1
    ------------------------------------------------------------  Cox-1b
473 TATCCCCAGCCCTCCAACCTACAACACAGCACATGACTACATCAGCTGGGAGTCCTTCTC  532

     N  V  S  Y  Y  T  R  I  L  P  S    Cox-1
    ----------------------------------   Cox-1b
533 CAACGTGAGCTACTATACTCGCATTCTGCCCTCT  566


    +++ intron-1
    *   translation stop codon
    --- untranslated region
```

Figure 1

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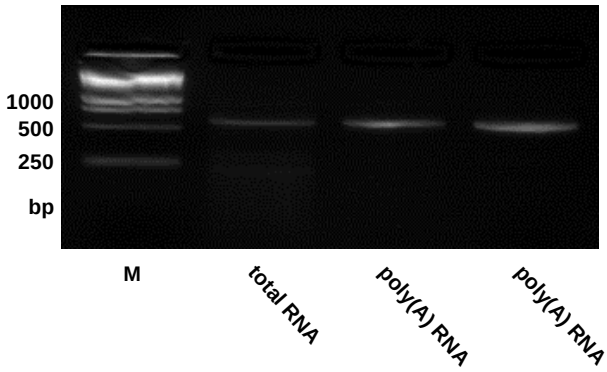

Figure 2

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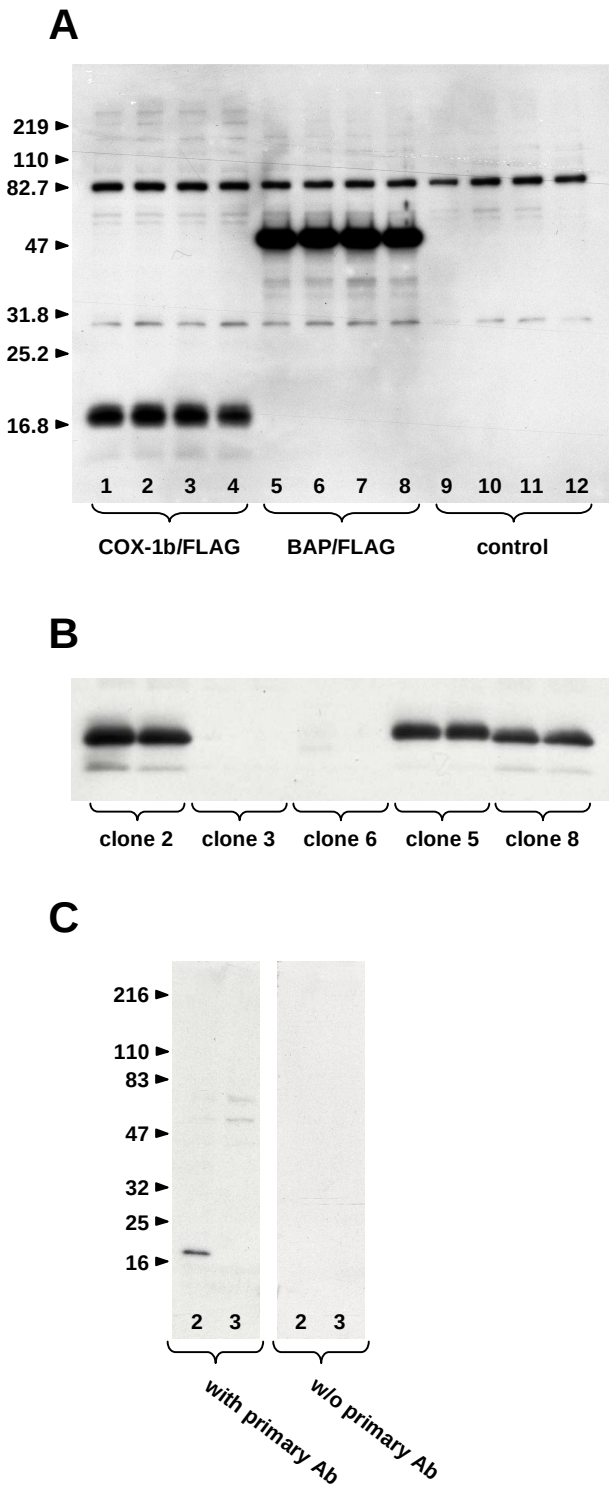

Figure 3

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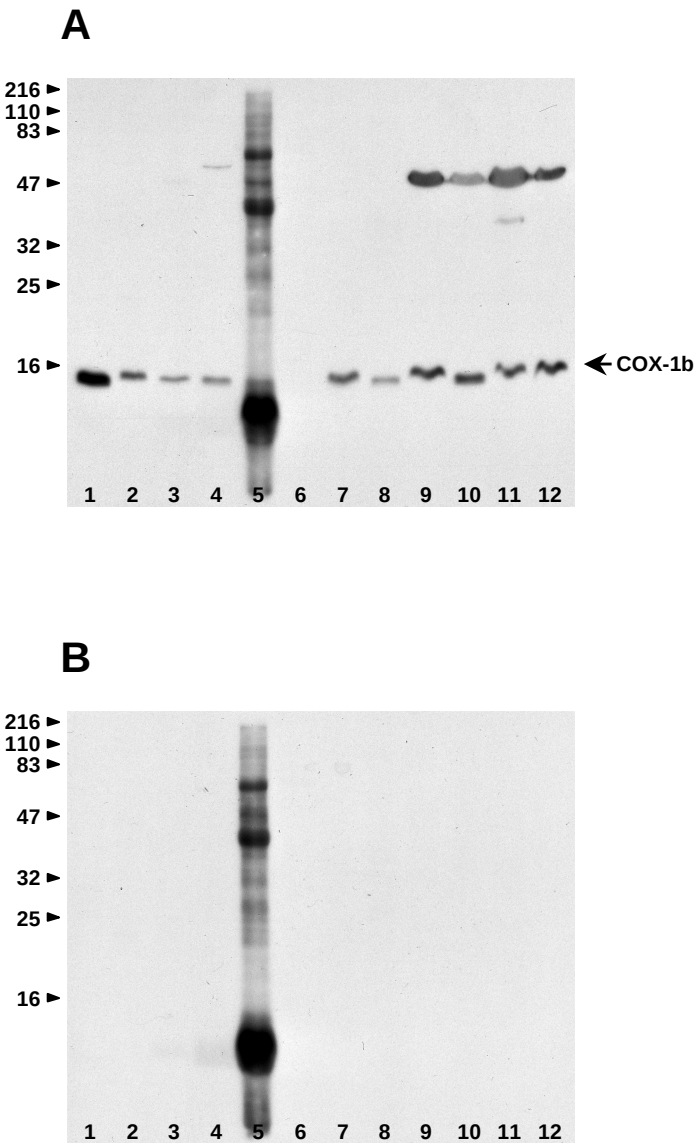

Figure 4

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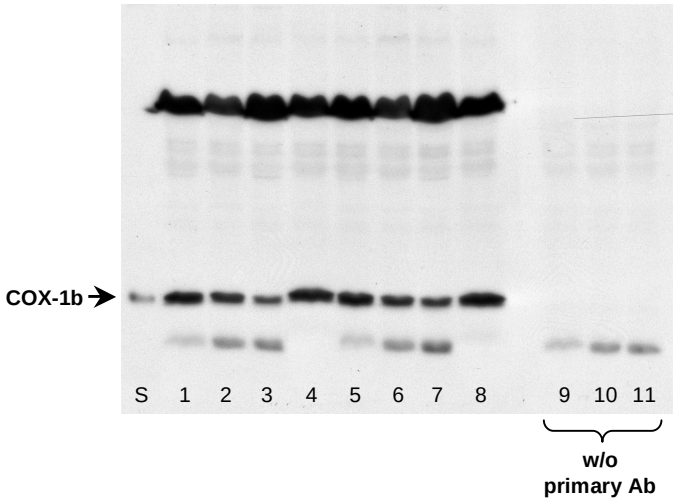

Figure 5

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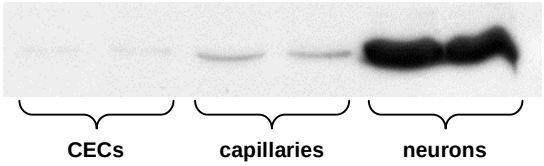

Figure 6

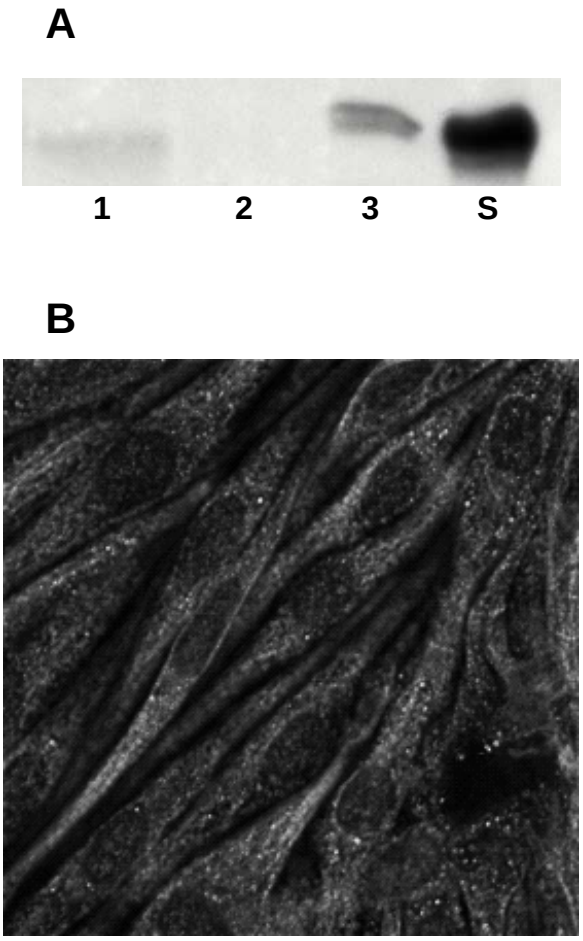

Figure 7

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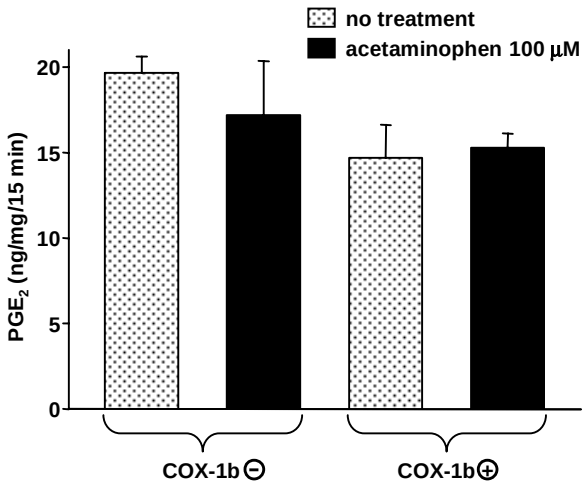

Figure 8

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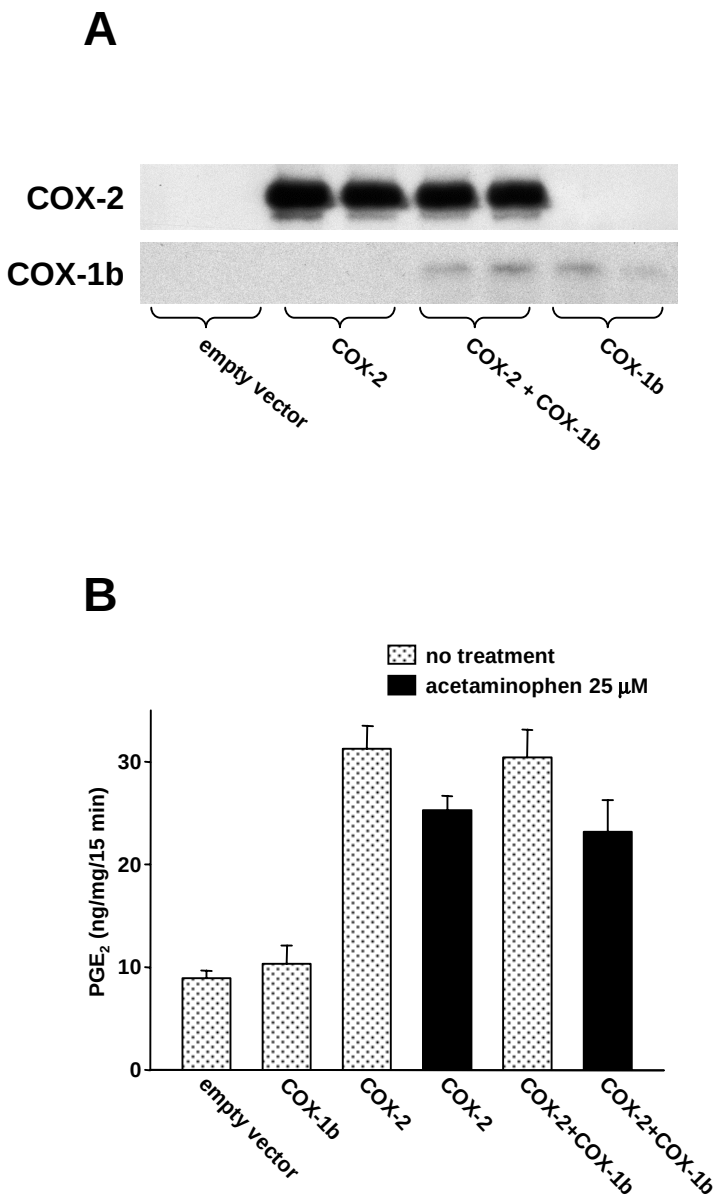

Figure 9