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Acetaminophen and the COX-3 Puzzle: Sorting out Facts, Fictions and Uncertainties

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Abbreviations:

COX: cyclooxygenase

NSAIDs: nonsteroidal anti-inflammatory drugs

PCOX: partial cyclooxygenase

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Abstract

Cyclooxygenase-3 (COX-3), a novel COX splice variant, was suggested as the key to unlocking the mystery of the mechanism of action of acetaminophen. While COX-3 might have COX activity in canine, and this activity might be inhibited by acetaminophen, its low expression level and the kinetics indicate unlikely clinical relevance. In rodents and humans COX-3 encodes proteins with completely different amino acid sequences than COX-1 or COX-2 and without COX activity; therefore it is improbable that COX-3 in these species plays a role in prostaglandin-mediated fever and pain. The aim of this review is an evaluation of the literature which seeks to point out critical theoretical and methodological limitations of the COX-3 studies which led several investigators to scientifically questionable conclusions.

Introduction

Acetaminophen is the best selling over the counter antipyretic and analgesic drug in the United States and it is also extensively used worldwide. Acetaminophen has similar antipyretic and analgesic potential to nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen, however, it is different from the other NSAIDs because it lacks antiinflammatory, antiplatelet and gastrotoxic activity. Since acetaminophen, unlike NSAIDs, has only a weak inhibitory effect on purified preparations of cyclooxygenase-1 (COX-1) and COX-2 at therapeutic concentrations, it has been believed that the target of acetaminophen is a "brain specific" COX enzyme which produces prostaglandins in the central nervous system thereby initiating fever and pain (Botting, 2000).

In 2002 Dan Simmons' group reported the discovery of a new COX isoenzyme, which was putatively the specific target of acetaminophen. The discovery of the so-called COX-3 as an acetaminophen-sensitive isoform seemed to solve the mystery of acetaminophen and attracted considerable attention, and several commentaries were published quickly in prominent scientific journals. This discovery in canine was generalized to humans and rodents without critical evaluation of the experimental data. Unfortunately, many in the scientific community now believe that the mechanism of action of acetaminophen is selective inhibition of COX-3. However, this hypothesis is not supported by experimental evidence. The aim of this review is a thorough evaluation of the scientific literature with particular examination of the critical theoretical and methodological contradictions of the COX-3 puzzle.

Canine COX-3

COX-3 was first described in dogs as an alternative splice variant of COX-1 (Chandrasekharan et al., 2002). Canine COX-3 mRNA is identical to the COX-1 mRNA except that intron 1 is retained. Two smaller forms of COX-1, partial COX or PCOX, have also been described (PCOX-1a and PCOX-1b). PCOX-1a contains intron 1 but lacks exon 5-8 while PCOX-1b is identical to PCOX-1a except that it does not contain intron 1. Since the normal start codon resides in exon 1 and the 90-nucleotide intron 1 sequence maintains the open reading frame, it is likely that the canine COX-3 mRNA creates a COX-1-related peptide containing a 30 amino acid insertion near the N-terminus (Chandrasekharan et al., 2002). The canine COX-3 peptide, if it exists normally, is expected to have COX activity because the majority of the sequence is identical to COX-1, except for the N-terminus, but this part of the molecule does not contribute to the enzymatic active center of the molecule (Simmons et al., 2004). To demonstrate whether the canine COX-3 and PCOX-1a mRNA would encode a functional protein, the authors cloned these COX-1 splice variants into a baculovirus expression vector and transfected Sf9 insect cells with the constructs. The authors reported that the insect cells produced the novel proteins and that canine COX-3 had prostaglandin E_2 (PGE_2) synthesizing capacity whereas PCOX-1a did not.

There are several methodological issues which should be considered. It was claimed that canine intron 1 shows a 75% sequence homology to intron 1 of either human or mouse COX-1 (Chandrasekharan et al., 2002). Alignment of the gene sequences utilizing open gaps makes this statement true (Fig. 1A). But if we align the sequences from the start codon without gaps, which is the actual reading frame for translation, the sequence homology is minimal (Fig. 1, 2). Consequently, the protein sequences encoded by intron-1 are completely different for the various species (Fig. 1B). Furthermore, Chandrasekharan *et al.* (Chandrasekharan et al., 2002) used an

oligopeptide corresponding to the first 12 amino acids of the predicted human COX-3 sequence for antibody production. But because of the above mentioned reason, there is only a 7-amino acid identity between the first 12 amino acids of human and canine COX-3 (Fig. 1B). Therefore, it is very unlikely that the antibody which was produced against the N-terminal part of the human COX-3 sequence will recognize the canine COX-3. Surprisingly, using the anti-human COX-3 antibody allowed them to detect COX-3 expression in insect cells which were transfected with the canine COX-3 sequence. Critical analysis of their results is difficult because they published only cropped parts of the Western blots of proteins from insect cells without corresponding molecular weight markers and these blots show several putative specific and non-specific bands. The same insect cell expression system was used to demonstrate the PGE_2 producing capacity of transfected cells as well as to determine the sensitivity of COX-3 to inhibition by different drugs (Chandrasekharan et al., 2002). Since the authors did not use mock-transfected insect cells as negative controls, did not provide Northern analysis or PCR as evidence of successful transfection, and because the anti-human antibody may not be appropriate to detect canine COX-3, there is a lack of convincing data indicating that the weak PGE_2 production of the insect cells transfected with COX-3 was derived from the enzymatic activity of canine COX-3.

According to Chandrasekharan *et al.* (Chandrasekharan et al., 2002) COX-3 is the specific target of acetaminophen; they found that the canine COX-3 had significantly higher sensitivity to acetaminophen than COX-1 or COX-2. Unfortunately, there are significant limitations to the interpretation of the results of their study. Isoforms of COX from dog (COX-3) and mice (COX-1, COX-2) were used and the basal enzyme activities (as measured by PGE_2 production from exogenous arachidonic acid) of the three constructs in Sf9 cells were significantly different. In addition, as it was mentioned above, the expression level of the canine

COX-3 construct in Sf9 cells is uncertain, because the authors used anti-human COX-3 antibody to detect the canine variant. The activity of the canine COX-3 construct was approximately 20% as compared to the mouse COX-1 construct and was only 4% as compared to the mouse COX-2 construct (Chandrasekharan et al., 2002). Boutaud *et al.* (Boutaud et al., 2002) showed that the sensitivity to acetaminophen depends on the level of peroxide generated in the solution and this varies with the COX enzyme activity, and indeed with the enzyme concentration. Acetaminophen apparently inhibits COX activity by reducing the oxidized form of the enzyme back to the catalytically inactive resting state and this occurs more readily when the enzyme activity and consequently the peroxide tone are low. Simply diluting the enzyme preparation has a major effect on the IC_{50} of acetaminophen (Boutaud et al., 2002). Therefore, it is difficult to know whether acetaminophen truly has a greater ability to inhibit COX-3 than COX-1 or COX-2, or if the IC_{50} values merely reflect the variable activities of the Sf9 constructs, as previously speculated by David Aronoff at a COX-3 forum in the internet (www.caymanchem.com/app/template/cox3,Home.vm). It is also important to mention that the IC_{50} values for acetaminophen in COX-3 transfected cells were 64 μM and 460 μM in the presence of 5 μM and 30 μM arachidonic acid, respectively (Chandrasekharan et al., 2002), whereas in other studies in endothelial cells (Boutaud et al., 2002; Kis et al., 2005), microglial cells (Fiebich et al., 2000; Greco et al., 2003) and in other cell types (for review see Graham and Scott, 2005), the reported IC_{50} values are several times lower. As it was also suggested by David Aronoff, using constructs with equivalent arachidonic acid utilization or purified preparations of the different COX isoforms exhibiting similar specific activities would allow a more useful comparison of the inhibitory potency of acetaminophen and NSAIDs against COX isoforms. Since the concentration of arachidonic acid in the incubation mixture also has a significant

influence on the inhibitory effect of acetaminophen (Boutaud et al., 2002), employing low concentrations of arachidonic acid and known natural stimulants of the prostaglandin cascade are required to characterize the selectivity of acetaminophen on the canine COX-3.

Human COX-3

The existence of a COX-1 splice variant which includes intron 1 has also been reported in humans (Chandrasekharan et al., 2002; Dinchuk et al., 2003; Cui et al., 2004; Qin et al., 2004). While the expression of COX-3 in other species is scientifically interesting, the value of COX-3 is as a potential target for drug development in people. Retention of intron 1 did not induce instability to the human COX-3 message, as it was evidenced by the half life of the COX-3 mRNA which was the same as that of COX-1 (Cui et al., 2004). Cui *et al.* described the presence of two stem-loop secondary structures in human intron 1 and suggested that these domains may play a role in regulating intron 1 splicing and in the determination of whether COX-1 or COX-3 signals are transcribed (Cui et al., 2004).

Whether the human COX-3 encodes a functional COX protein is questionable. The retention of intron 1, which is 94 nucleotides in humans, should lead to a shift in the reading frame, premature termination and a truncated protein. Dinchuk *et al.* (Dinchuk et al., 2003) cloned 24 COX-1 cDNAs from a human cerebral cortex cDNA library and observed that 7 of the 24 clones retained intron 1 and in all such a clones intron 1 was 94 nucleotides long thus leading to out of frame mutation. Therefore the predicted amino acid sequence of the human COX-3 protein is completely unlike COX-1 with a calculated molecular weight (MW) of only 8.7 kDa. Chandrasekharan *et al.* (Chandrasekharan et al., 2002) demonstrated a barely detectable band with an approximate MW of 65 kDa with Western blot analysis using anti-human COX-3

antibody or anti-goat COX-1 antibody in human aortic tissue. They believed that this band represents the human COX-3 protein. This finding was explained with the hypothesis that a ribosomal frameshift corrected the sequence into the regular open reading frame of the COX-1 leading to the synthesis of a peptide which is similar to COX-1 except that it is 31 amino acids longer in length. Although many RNA viruses employ -1 frameshifting and a widespread involvement of -1 frameshifting in eukaryotic gene expression seems likely as judged from computer-assisted database screens (Hammell et al., 1999), there is only one example to date that this occurs in an eukaryotic gene, the mouse *Edr* (embryonal carcinoma differentiation regulated), but even this signal appears to be a relic of retroviral origin (Shigemoto et al., 2001). The presence of the two stem-loop structures in intron 1 also would not explain a possible -1 shift in the reading frame (Cui et al., 2004).

There is another important question regarding the Western blot results on human aortic tissue. Chandrasekharan *et al*. claimed that they detected human COX-3 as a 65 kDa protein (Chandrasekharan et al., 2002). If we accept the hypothesized -1 frameshifting during COX-3 translation, the retention of intron 1 would lead to an insertion of additional 31 amino acids into the COX-1 sequence, and thus it would be expected that COX-3 protein runs larger than unglycosylated COX-1, which is 68.5 kDa. In addition, they demonstrated that the canine COX-3 was glycosylated in transfected Sf9 cells, therefore it is likely that the human COX-3 would also be glycosylated which would further increase its molecular weight. Cui *et al.* (Cui et al., 2004) also reported that they detected human COX-3 protein in human hippocampal tissues and human neural cells (positive for glial and neuronal markers) with Western blot analysis using anti-human COX-3 antibody, but they did not give any specific information regarding the size of the detected protein and whether one or many bands were detected.

Qin *et al.* (Qin et al., 2004) amplified the unique N-terminal cDNA fragment of human COX-3 from several human cDNA libraries and reported the existence of three splice variants in human tissues; the most prevalent variant was where the whole 94 nucleotide-long intron was retained leading to the shift of the reading frame and the synthesis of a truncated peptide (Qin et al., 2004). The other two variants retained almost the entire intron 1, but were missing a nucleotide in one of two different positions, thereby encoding full length and enzymatically active COX molecules. However, the expression level of these splice variants compare to the level of COX-1 was very marginal. The full length splice variants catalyzed the synthesis of $PGF_{2\alpha}$ from arachidonic acid, but there was no preferential selectivity for inhibition by selected NSAIDs or acetaminophen (Qin et al., 2004).

COX-3 in rodents

COX-3 mRNA has been detected in cultured cells of the rat central nervous system (Kis et al., 2003) and in tissues from rat (Kis et al., 2004; Warner et al., 2004) and mouse (Shaftel et al., 2003; Ayoub et al., 2004; Dou et al., 2004). In particular, we have shown that COX-3 mRNA is relatively abundant in cultured cerebral endothelium (Kis et al., 2003) as well as in freshly harvested cerebral microvessels (Kis et al., 2004) of rat. Similar to our findings, Cui *et al.* detected the highest COX-3 mRNA expression in the cerebral vasculature in human brain (Cui et al., 2004).

The most important issue again is whether the COX-3 mRNA in rodents encodes a functional COX-3 protein. The situation in rodents is similar as in humans; the retention of intron 1, which is 98 nucleotides, in rat and mouse, 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.

Shaftel *et al.* (Shaftel et al., 2003) mistakenly reported that in mouse the intron 1 is 102 nucleotide-long and it keeps the sequence in frame. It is theoretically 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 COX-1 variant is produced. According to our latest results this is not the case. We cloned and sequenced rat and mouse COX-3 mRNA and our sequence analysis indicated that the 98-nucleotide intron 1 of the COX-1 gene remains unprocessed in the COX-3 mRNA causing a frameshift mutation with premature termination and a 127-amino acid open reading frame with no sequence similarity with known cyclooxygenases (Snipes et al., 2005). The rat and mouse COX-3 mRNA shows 93 % homology (Snipes et al., 2005). Transient and permanent transfection of COS-7 cells with a vector containing the rat COX-3 cDNA resulted in synthesis of a protein of the expected size. We generated an affinity-purified polyclonal antibody against the rat COX-3 protein. A peptide corresponding to the N-terminal 2-17 amino acids of rat COX-3 mRNA encoded protein (SRESDPSGAPTRPGIR), as predicted by cDNA sequences, was used to induce antibody production. This part of the COX-3 molecule is 100% identical in rat and mouse. Western blot analysis of rat tissues using this antibody demonstrated the existence of rat COX-3 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-3 does not have cyclooxygenase activity and does not have any effect on the inhibition of prostaglandin production of COX-2 by acetaminophen (Snipes et al., 2005). In support our findings, Warner *et al.* (Warner et al., 2004) demonstrated in a variety of rat tissues that the production of prostanoids is dependent on the two known functional isoforms of cyclooxygenase, COX-1 and COX-2, and that there is no evidence for the involvement of a particular acetaminophen-sensitive COX-3 isoform.

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On the other hand, Ayoub et *al* (Ayoub et al., 2004) reported that acetaminophen-induced hypothermia in rats occurs via inhibition of COX-3. They found that in wild type and in COX-2$^{-/-}$ mice, acetaminophen (300 mg/kg) produced hypothermia accompanied by a reduction in brain PGE_2 levels, whereas in COX-1$^{-/-}$ mice, the hypothermia to this dose of acetaminophen was attenuated. The brains of COX-1$^{-/-}$ mice had significantly lower levels of PGE_2 than those of wild type animals, and these levels were not reduced further by acetaminophen. From these results they proposed that acetaminophen is a selective inhibitor of COX-3 and this enzyme is involved in the continual synthesis of PGE_2 that maintains a normal body temperature (Ayoub et al., 2004). Taking the fact that rat and mouse COX-3 has a completely different amino acid sequence than COX-1 and COX-2 and it does not have cyclooxygenase activity, the result of Ayoub et *al* (Ayoub et al., 2004) is surprising. In addition, although COX-3 is a splice variant of COX-1, it does not mean that the COX-1$^{-/-}$ mouse is a COX-3$^{-/-}$ mouse. In the COX-1$^{-/-}$ mouse the C terminal part of COX-1 is disrupted by gene targeting (Langenbach et al., 1995). The targeting vector was designed to replace 1 kilobase of intron 10 together with the splice junction and first 44 bp of exon 11 with the neomycin resistance gene (Langenbach et al., 1995). If a protein were made from the resulting disrupted gene, it would lack only the C-terminal 120 acids which are crucial to the enzyme activity of COX-1. However, the disrupted COX-1 gene contains the entire sequence for the COX-3 protein. We isolated total RNA from COX-1$^{-/-}$ mice from the same strain (Taconic, Germantown, NY) (Langenbach et al., 1995) used by Ayoub et *al* (Ayoub et al., 2004). Our RT-PCR analysis showed that COX-3 mRNA was transcribed in COX-1$^{-/-}$ mouse, moreover COX-3 protein was detected with Western blot analysis (Fig. 3). Ayoub et al (Ayoub et al., 2004) reported COX-1, COX-2, and COX-3 mRNA expression data in wild-type , COX-2$^{+/-}$, and COX-2$^{-/-}$ mice but did not show equivalent data from COX-1$^{+/-}$ and COX-

$1^{-/-}$ mice, however, these strains played a crucial part in their study. Using the COX-$1^{-/-}$ mouse, it was an unsubstantiated conclusion that acetaminophen induces hypothermia in rats via inhibition of COX-3 (Ayoub et al., 2004). In addition, the primer set what they used to detect COX-3 mRNA was also specific for PCOXa (Ayoub et al., 2004). Thus the signal that was denoted as COX-3 in the RT-PCR also included PCOXa.

Dou *et al.* (Dou et al., 2004) used the same COX-$1^{-/-}$ mice and the same primer set to detect COX-3 mRNA in their experiments as Ayoub et *al* (Ayoub et al., 2004), and similarly they published all other results, except their COX-3 RT-PCR data in COX-$1^{-/-}$ mice. They found COX-3 protein expression in dorsal root ganglia of wild type mice with immunohistochemistry but not with Western blot analysis. This is an interesting finding, because Western blot analysis can be more sensitive to detect proteins than immunohistochemistry and it is more specific because the separation of proteins based upon molecular weight. A possible explanation is that the COX-3 immunohistochemistry resulted in a false positive result due to non-specific binding, a phenomenon which is not unusual with polyclonal antibodies. A more serious limitation to the interpretation of their results is the use of an inappropriate antibody. The 13-amino acid sequence (MSREFDPEAPRNC) which was used to generate the anti-mouse COX-3 in the original study (Chandrasekharan et al., 2002) also is used by companies which are commercializing anti-mouse COX-3 antibodies (see Table 1 in (Davies et al., 2004)). However, this sequence is substantially (more that 50%) different from the actual first 13 amino acids of the mouse or rat COX-3 (MSRESDPSGAPTR). Therefore it is unlikely that those antibodies will recognize the mouse or rat COX-3 peptide.

COX splice variants

The alternative splicing of mRNA is a significant source of generating proteome diversity through its ability to control the expression of proteins (Modrek and Lee, 2002). Alternative splicing can regulate protein function, for instance by the removal of interaction or localization domains, and also can influence gene expression by splicing transcripts into unproductive mRNAs targeted for degradation. It has been estimated that at least half of all human genes utilize alternative RNA processing to generate multiple mRNA products (Modrek and Lee, 2002).

Several splice variants of COX-1 and COX-2 enzymes have been reported. A splice variant of COX-1 lacking exon 9 was reported in human fibroblasts (Diaz et al., 1992) and recently it was shown that the mRNA transcript for this COX-1 variant is present in various human tissues, however, the corresponding protein is either not formed or subject to rapid proteolytic degradation (Schneider et al., 2005). Yet another COX-1 splice variant containing an intronic sequence was described in rat tracheal epithelial cells (Kitzler et al., 1995), in rat stomach (Vogiagis et al., 2000) and in rat colorectal tumors (Vogiagis et al., 2001). Another COX-1 splice variant was shown to be present in endothelial cells (Hla, 1996). A COX-2 splice variant with retention of the intron between exons 1 and 2 was described in chicken embryo fibroblasts (Xie et al., 1991) and a COX-2 splice variant with an unspliced intron between exon 7 and 8 was reported in human myometrium (Huang et al., 2003). A COX-2 splice variant was also found in platelets (Censarek et al., 2004). Plant and Laneuville (Plant and Laneuville, 1999) described three transcripts of COX-1 gene as well as two transcripts of the COX-2 gene. These studies suggest that several splice variants of both COX-1 and COX-2 exist (Davies et al., 2004). At this point the physiological role of the different COX splice variants is not known and it seems that COX-3 is just another COX splice variant among many others.

COX-3 is a potential target for nonsense-mediated decay

Nonsense-mediated mRNA decay is a surveillance pathway that reduces errors in gene expression by eliminating a variety of aberrant mRNAs that arise as a result of either mutations or defects in pre-mRNA processing (Baker and Parker, 2004). One of the primary targets of nonsense-mediated decay is mRNA with out-of-frame mutations which lead to premature translation termination. In mammalian mRNAs, stop codons at distances greater than 50–55 nucleotides upstream from the nearest 3′ exon–exon junction are perceived as premature and lead to nonsense-mediated decay (Nagy and Maquat, 1998). According to the sequence analysis of the rat COX-3 mRNA and the predicted human COX-3 mRNA the distance between the stop codon and the next exon-exon junction are 74 and 56 nucleotides, respectively, thus these transcripts are potentially targeted by nonsense-mediated decay. The nonsense-mediated decay does not provide complete elimination of all premature stop codon containing mRNAs, 10-30% of nonsense transcripts can escape degradation (Perrin-Vidoz et al., 2002) and there are examples of protein synthesis from mRNAs normally subjected to nonsense-mediated decay (Dreumont et al., 2005). All available experimental data shows that the expression levels of COX-3 in humans, rats and mice are only a few percent of the levels of COX-1 expression in these species, a result consistent with the hypothesis that COX-3 mRNAs are subject to nonsense-mediated decay in rodents and man.

What is the target of acetaminophen?

Acetaminophen has potent antipyretic and analgesic potential but has only weak anti-inflammatory activity (Botting, 2000). Thirty years ago Flower and Vane reported that prostaglandin production in brain was 10 times more sensitive to inhibition by acetaminophen

than that of in spleen (Flower and Vane, 1972). It is a generally accepted concept that acetaminophen acts centrally and it is a weak inhibitor of prostaglandin synthesis by COX-1 and COX-2 (Botting, 2000). This commonly accepted statement, however, is not supported by the literature.

In a very recent comprehensive review Graham and Scott (Graham and Scott, 2005) critically examined the available literature. They showed that although acetaminophen is a weak inhibitor of prostaglandin synthesis of COX-1 and COX-2 in broken cell systems (Swierkosz et al., 2002), therapeutic concentrations of acetaminophen significantly inhibit prostaglandin synthesis in intact cells and tissues as was reported by several groups (for review see Graham and Scott, 2005). Graham and Scott pointed out that acetaminophen has similar pharmacological effects to selective COX-2 inhibitors and it is likely that the target of acetaminophen is COX-2 (Graham and Scott, 2005). Our latest results also support the COX-2 hypothesis. We found that acetaminophen dose-dependently inhibited both basal and lipopolysaccharide (LPS)-induced PGE_2 production in cerebral endothelial cells with IC_{50} values of 15.5 μM and 6.9 μM, respectively (Kis et al., 2005). LPS stimulation increased the expression of COX-2 but not COX-1 or COX-3. In addition, the selective COX-2 inhibitor, NS398, was equally as effective as acetaminophen in blocking LPS-induced PGE_2 production (Kis et al., 2005). Our results suggest that acetaminophen acts against COX-2 and not COX-1 or COX-3. Because increased prostaglandin production in cerebral endothelial cells plays a crucial step in fever development (Zhang and Rivest, 2003) our findings support a critical role for cerebral endothelium in the antipyretic action of acetaminophen.

The molecular mechanism of action of acetaminophen has not been clearly demonstrated. It has been suggested that acetaminophen reduces the oxidized form of the COX back to the

catalytically inactive state and consequently low levels of oxidants potentiate this inhibition while high levels decrease this effect (Ouellet and Percival, 2001; Boutaud et al., 2002). Therefore, the weak anti-inflammatory activity of acetaminophen could be due to its poor ability to inhibit COX in inflamed tissue where the peroxide level is high. In contrast, the unique strictly controlled environment of the brain may favor the effect of acetaminophen (Flower and Vane, 1972). However, this view cannot explain why prostaglandin production in astrocytes, which possess the highest anti-oxidant levels in the brain, is less responsive to acetaminophen (Lanz et al., 1986) than in microglia, which produce large amounts of reactive oxygen species and are very sensitive to acetaminophen even after LPS stimulation (Greco et al., 2003). Moreover, Swierkosz *et al.* (Swierkosz et al., 2002) and Warner *et al.* (Warner et al., 2004) were not able to demonstrate in the rabbit, rat and mouse that COX in the brain is more sensitive to acetaminophen than in other tissues.

Conclusions

The discovery of COX-3 in canine seemed to offer a key to unlock the long sought after mechanism of action of acetaminophen. Unfortunately the situation is not entirely clear but most experimental data lead us to conclude that the so-called COX-3 is just another COX-1 splice variant. Therefore, it should be named accordingly. We (Snipes et al., 2005) and others (Davies et al., 2004) believe that the name COX-3 should be reserved for the product of an independent third cyclooxygenase gene, which has not yet been identified. We endorsed the term COX-1b, instead of COX-3, which better reflects the true relationships among the cyclooxygenase variants (Snipes et al., 2005).

COX-3 might have cyclooxygenase activity in canine and this activity might be inhibited by acetaminophen, however, its low expression level and the available kinetic data indicate that this selective interaction is unlikely to be clinically relevant. Although several years have passed since the original publication (Chandrasekharan et al., 2002), there is no any follow-up study which would confirm the COX activity of the canine COX-3 and its sensitivity to acetaminophen.

In human and rodents the mRNA transcript of COX-3 is potentially targeted by nonsense-mediated decay, which explains its low expression level. The function of the COX-3 protein is currently unknown. COX-3 proteins from dog, human and rodents do not show substantial homology (Fig. 2), which would be expected from a protein with physiological function. Although COX-3 encoded proteins were also detected in rodents (Snipes et al., 2005) and in human (Qin et al., 2004), we do not think that in these species they have functional importance, but rather are simply the product of an inefficient nonsense-mediated decay of a COX-1 splice variant. Because the protein encoded by human and rat COX-3 has a completely different amino acid sequence than COX-1 and COX-2 and it does not have cyclooxygenase activity we named it as **C**yclo**O**xygenase **VA**riant **P**rotein (COVAP) to distinguish it from the known prostaglandin synthesizing COX isoforms (Snipes et al., 2005).

The results from dog were transferred to humans and rodents without critical evaluation of the data. But evidence does not support the claims (Chen and Bazan, 2003; Ehrlich et al., 2004; Gryglewski et al., 2005) that acetaminophen is a selective COX-3 inhibitor in rodent studies. As it was previously highlighted by Davies et al. (Davies et al., 2004) the use of this term has been undertaken without any proof of a functional rat COX-3 protein or that acetaminophen works selectively through such an enzyme *in vivo* in any species. It is also important to note that commercially available anti-COX-3 antibodies can only be used in a

species-selective manner because of considerable differences among amino acid sequences of COX-3 from different species. In addition, as we mentioned above, the amino acid sequence of the hapten peptide used to generate the anti-mouse COX-3 antibodies is substantially different from the corresponding sequence of the mouse COX-3, therefore these antibodies are inappropriate to detect the mouse COX-3 peptide.

The exact mechanism of acetaminophen still remains a mystery, but because much information has already been available about COX splice variants, we believe that studying the transcription of COX genes might help us to better understand the function of alternative splicing in eukaryotes. In some prior publications on COX-3 the authors did not critically evaluate essential methodological and theoretical issues, or consider the relevance of species differences in the COX-1 gene sequence and intron structure. This review might lead to the reevaluation of previous data from COX-3 research.

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Legends to the Figures

Figure 1

Partial sequences of human and canine COX-3 cDNA

A: Alignment of human and canine intron 1 sequences with open gaps. This alignment shows 76% homology between the human and canine intron 1 sequences. Identical nucleotides are with bold letters.

B: Alignment of human and canine COX-3 open reading frames from the start codon to the end of intron 1 and their translation into amino acid sequences. Exon-1 encoded sequences are underlined. Identical nucleotides and amino acids are with bold letters.

Figure 2

Amino acid sequences of COX-3 in human, dog, rat and mouse

The COX-3 is 82 amino acids in human, 633 in dog and 127 in rat and mouse. The canine sequence shows only the first 150 amino acids. Sequence encoded by intron 1 is underlined.

Figure 3

COX-1 and COX-3 mRNA and protein expression in COX-1$^{-/-}$ and COX-1$^{+/+}$ mice

A: Total RNA was isolated from kidneys of COX-1 knockout (COX-1$^{-/-}$) and COX-1 wild type(COX-1$^{+/+}$) mice (Taconic, Germantown, NY). The primer set for RT-PCR to detect COX-3 was designed as described previously (Snipes et al., 2005). Our COX-1b primer set distinguishes between COX-3 and COX-1, and it also distinguishes between COX-1b and “partial” cyclooxygenases (PCOX-1a, PCOX-1b). The detect COX-1, the antisense primer was designed

to bind a 3' terminal part of the mRNA which is disrupted in the COX-1$^{-/-}$ mouse. The expected molecular weights of the PCR products were 573 bp for COX-3, 344 bp for COX-1 and 285 for b-actin. M: molecular weight marker; 1, 2: COX-1 knockout mice; 2, 4: COX-1 wild type mice. B: Protein was isolated from kidneys of COX-1 knockout and COX-1 wild type mice. Western blot analysis was carried out to determine the expression levels of COX-1, COX-2 and β-actin using specific antibodies. 1-3: COX-1 knockout mice; 3-6: COX-1 wild type mice.

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A

```
human    8 GTGAGTGCGACCCCGGTGCCCGGTGGGGAATTTTCTTGGCCTCCTGG 54
dog      8 GTGAGTTCGACCCTGAGGCCCCCAGG..AACCCTCTTCGCCTCCCGG 52

human   55 TGGAGCCTTGAATGCCAGGCTCAGCCCCTCATCTCTCTCCTCTGCAG 101
dog     53 GGGAGCCTCGAATGCCAGGCCCAG.CCCTCACCTCTC.GCTCCGCAG 97
```

B

```
human    1 ATGAGCCGTGAGTGCGACCCCGGTGCCCGGTGGGGAATTTTCTTGGCCTCC 51
           M  S  R  E  C  D  P  G  A  R  W  G  I  F  L  A  S

dog      1 ATGAGCCGTGAGTTCGACCCTGAGGCCCCCAGGAACCCTCTTCGCCTCCCG 51
           M  S  R  E  F  D  P  E  A  P  R  N  P  L  R  L  P

human   52 TGGTGGAGCCTTGAATGCCAGGCTCAGCCCCTCATCTCTCTCCTCTGCAG 101
           W  W  S  L  E  C  Q  A  Q  P  L  I  S  L  L  C  R

dog     52 GGGGAGCCTCGAATGCCAGGCCCAGCCCTCACCTCTCGCTCCGCAG      97
           G  E  P  R  M  P  G  P  A  L  T  S  R  S  A  G
```

Figure 1

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```
human    1 MSRECDPGARWGIFLASWWSLECQAQPLISLLCRESLALVLAVPAPAPAA 50
dog      1 MSREFDPEAPRNPLRLPGEPRMPGPALTSRSAGGSRLHRWPLLLLLLLLL 50
rat      1 MSRESDPSGAPTRPGIRWPAGGALNVRLNSLFPLQEGVSRSSFPCCCSCC 50
mouse    1 MSRESDPSGAPTRPGIRWPAGGALNARLNSLFLLQEGVSRSGFPCCCSCC 50

human   51 PRPARGPRGAHASESLLLLSMPAPGHLCPLRP 82
dog     51 PPPPVLPAEARTPAPVNPCCYYPCQHQGICVRFGLDRYQCDCTRTGYSGP 100
rat     51 CSHHPRYCSQMLGYPHQSIPVVTIHARTRVSVSASASTTTNVTVLARATR 100
mouse   51 CRRHPRSCSQILGCPHQSIPVVTIRARTRVSVSALASTTTSVIVLARATQ 100

dog    101 NCTIPELWTWLRNSLRPSPSFLHFLLTHGRWFWEFINATFIRDMLMRLVL 150
rat    101 APTVLSLRSGPGFGVPCGPAPHSPISC 127
mouse  101 APTVPSLRSGPGFGILCGPAPRSPISC 127
```

Figure 2

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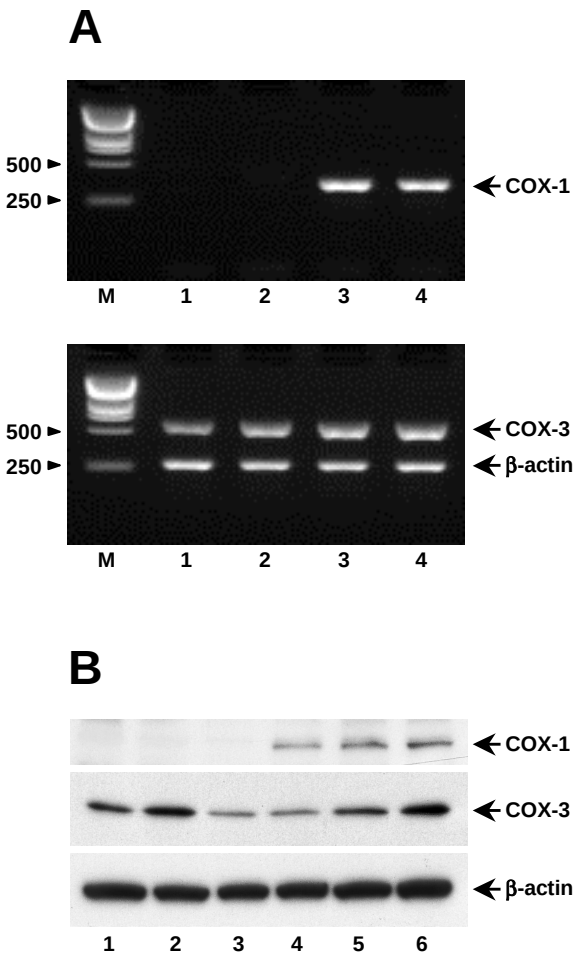

Figure 3