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Gene Expression and Immunochemical Localization of Major Cytochrome P450 Drug Metabolizing Enzymes in Bovine Nasal Olfactory and Respiratory Mucosa

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Abstract

Despite tremendous advancement in the characterization of nasal enzyme expression, knowledge of the role of the nasal mucosa in the metabolism of xenobiotics is still inadequate, primarily due to the limited availability of *in vitro* models for nasal metabolism screening studies. An extensive knowledge of the oxidative and conjugative metabolizing capacity of the cattle (*Bos taurus*) olfactory and respiratory mucosa can aid in efficient use of these tissues for pre-clinical investigations of the biotransformation and toxicity of therapeutic agents following nasal administration or inhalation. Cows are also exposed to a variety of airborne pollutants and pesticides during their lifetime, the metabolism of which can have profound toxicological and ecological consequences. The aim of the present study was to characterize cytochrome P450 (CYP) enzyme expression in the bovine nasal mucosa. Amplification of the specific genes through real time RT-PCR confirmed expression of several CYP enzymes in bovine hepatic and nasal tissues. The results demonstrate that bovine nasal olfactory and respiratory mucosal and liver tissues express similar populations, families, and distributions of CYP enzymes, as has been previously reported with other species, including humans. Bovine *ex vivo* tissues can serve as a readily available reference tissue to elucidate preclinical toxicokinetic effects resulting from exposure to substances in the environment or following drug administration.

Keywords

Nasal Mucosa; Metabolism; Cytochrome P450; Gene Expression; Immunohistochemistry; Real Time-PCR

1. Introduction

Pre-systemic elimination by local enzymatic degradation is one of the key barriers to the transport of xenobiotics across the nasal mucosa. The nasal olfactory and respiratory

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Declaration of Interest

The authors report no conflict of interest in the work described in this report.

mucosae have been well documented to host a diverse array of phase I and II drug metabolizing enzymes (Gu et al., 2000; Heydel et al., 2013; Legendre et al., 2014; Thiebaud et al., 2013, 2010; Thornton-Manning, 1997; Watelet et al., 2009; Wong and Zuo, 2010; Zhang et al., 2005), some of which have been implicated in tissue-specific toxicological reactions. An increasing number of reports of the induction of nasal tumors or lesions in laboratory animals following inhalation of compounds undergoing metabolic activation to toxic compounds fueled interest in the area of enzymatic barrier properties of the nasal mucosa (Brittebo, 1997; Dahl and Hadley, 1991; Ding and Kaminsky, 2003; Reed, 1993; Thornton-Manning, 1997). The activity of nasal enzymes in metabolizing intranasally administered therapeutic agents during their transfer across the nasal mucosal layers, and the toxicological consequences of those metabolic reactions on the structural organization and integrity of the nasal mucosa, have not yet been carefully studied. The nasal olfactory and respiratory mucosae of rodents and humans have been relatively well characterized for enzyme expression (Chen, 2003; Gu et al., 2000; Ling et al., 2004; Marini et al., 2001; Minn et al., 2005; Su et al., 1996; Zhang et al., 2005). In contrast, however, the nasal mucosa of cattle has received relatively less attention.

Cows are inexpensive and easily accessible sources of *ex-vivo* tissues in the United States. The use of bovine tissues for studying the enzymatic activity in various organs, such as the liver, lungs, intestinal mucosa, kidneys, coronary arteries, and tongue, has become increasingly popular in recent years (Darwish et al., 2010(a), (b); Maté et al., 2015; Virkel et al., 2010; Zancanella et al., 2010). The proteolytic capacity of the bovine nasal mucosa has been used to investigate peptide transport and metabolism across the nasal mucosa (Lang et al., 1998, 1996; Schmidt et al., 2000). However, research reports characterizing the CYP-dependent monooxygenases in cattle are still scarce. In rodents and laboratory animals, the small size of the nasal cavity and the larger surface area coverage of the olfactory mucosa make it virtually impossible to effectively distinguish between the olfactory and respiratory mucosa. However, in cattle, the size of the nasal cavity is large and the organization of the nasal mucosa resembles that in humans, facilitating distinction and easy preparation of the olfactory and respiratory mucosa (Smith and Bhatnagar, 2004).

Evaluation of the metabolic capacity of the bovine nasal mucosa is critical for several reasons. An extensive knowledge of the expression and activity of the oxidative and conjugative drug metabolizing capacity of the bovine olfactory and respiratory mucosa can aid in efficient use of these tissues for high-throughput *in vitro* screening of the biotransformation of therapeutic agents during transport across the nasal mucosa. An improved understanding of cattle nasal biotransformative capacity is also essential to evaluate the risk of contamination of dairy or meat products with potentially toxic metabolites from chronic inhalation of pesticide or other airborne chemicals. In addition, knowledge of the distribution and activity of the nasal enzymes can be used to evaluate the effects of enzyme inhibitors and inducers on overall biotransformative capabilities of the nasal mucosa.

The aim of the present study was to investigate the metabolic capability of the bovine nasal mucosa through a screening of CYP gene expression and tissue-specific protein localization. Using quantitative RT-PCR, the relative expression levels of major CYP enzymes in the

bovine olfactory and respiratory mucosae were determined, and the gene expression of the enzymes in the bovine liver was also examined and compared with that in the nasal tissues to better estimate the metabolic barrier presented by the nasal tissues. The nomenclature used for bovine CYP enzymes studied using RT-PCR in this study is given in Table I along with their NCBI accession numbers (Edgar et al., 2002). Localization of the major CYP enzymes in the bovine olfactory and respiratory explants was examined by immunofluorescence staining to confirm the translation of gene sequences into the encoded proteins and to determine the distribution of those enzymes within the nasal tissues.

2. Materials and Methods

2.1 Materials

For PCR reactions, TRIzol[®] reagent for RNA extraction and SuperScript[®] III reverse transcriptase for cDNA synthesis were purchased from Invitrogen (Life Technologies[™], Carlsbad, CA). Oligonucleotide primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA). Real-time qPCR Master Mix SYBR[®] Advantage[®] was purchased from Clontech Laboratories, Inc. (Mountain View, CA). MicroAmp[®] Fast optical 48-well reaction plates were obtained from Applied Biosystems[®] (Life Technologies[™], Carlsbad, CA), and the thermal adhesive sealing film to cover the PCR plates was purchased from Research Products International Corp. (Mount Prospect, IL). QIAquick[®] gel extraction kit was purchased from Qiagen Inc. (Valencia, CA).

Bovine CYP specific monoclonal antibodies are not commercially available. Hence antibodies cross-reactive to bovine proteins were obtained. Primary antibodies, including mouse monoclonal anti-human CYP2A6 (AB56069) and mouse monoclonal anti-human CYP1A2 (AB22717), were obtained from Abcam Inc. (Cambridge, MA). Rabbit polyclonal anti-human antibody to CYP 2C8, 2C9, 2C19 (CR 3280), and rabbit monoclonal anti-human antibody to CYP3A4 (CR 3340) were obtained from Biomol International LP (Plymouth Meeting, PA). Alexa fluor 488 conjugated secondary antibodies, goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG as well as horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, the nuclear stain ToPro3, and Dulbecco's phosphate buffered saline (PBS) were obtained from Molecular Probes[®] (Life Technologies[™], Carlsbad, CA). All other reagents were obtained from Sigma Chemical (Sigma-Aldrich, St. Louis, MO).

2.2 Preparation of animal tissues

Bovine (*Bos taurus*) (six animals, approximately two years old) nasal explants and liver specimens were obtained from and dissected at Bud's Custom Meats Co. (Riverside, IA). The nasal respiratory and olfactory mucosae were collected by making incisions along the lateral walls of the nasal cavity and a horizontal incision along the ocular plane. The ethmoturbinates, covered by the olfactory mucosa, and the maxilloturbinates, covered by the respiratory mucosa, as well as liver tissues, were excised immediately after death. For histological and immunofluorescence examinations, the excised olfactory and respiratory mucosal sections were fixed immediately in zinc formalin (1% zinc sulfate in 3.7% unbuffered formalin) for 36 hours. For RNA extraction, the nasal and liver tissues were

frozen in liquid nitrogen immediately after dissection and were kept frozen at -80°C until use.

2.3 RNA extraction

RNA extraction and real-time RT PCR were carried out at the College of Pharmacy, University of Iowa (Iowa City, IA). Total RNA was isolated from frozen tissues using TRIzol[®] reagent according to the manufacturer's instructions. Briefly, 1 mL of TRIzol[®] was added to a small piece of frozen tissue and homogenized at 4°C to release the nucleic acids. The RNA was isolated using a phenol-chloroform extraction method provided in the TRIzol[®] kit. The concentration and purity of RNA was checked by absorbance spectroscopy using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The integrity of the RNA samples was assessed by denaturing electrophoretic analysis on 1% agarose gels stained with ethidium bromide using FisherBiotech electrophoresis system (Fisher Scientific, Pittsburgh, PA).

2.4 Reverse transcription

Complementary DNA (cDNA) was synthesized from RNA samples using a SuperScript[®] III reverse transcriptase kit in a Bio-Rad MyCycler[™] thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA). The reaction was performed at 50°C for 90 minutes with the use of 2.5 μg of total RNA, 200 units of SuperScript[®] III reverse transcriptase enzyme, 2.5 μM oligo(dT) primer, and 5 mM MgCl_2 in the presence of 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM dithiothreitol in a total volume of 20 μL . The reverse transcriptase was inactivated by heating to 70°C for 15 minutes. The quality of the synthesized cDNA was confirmed by denaturing agarose gel electrophoresis on 1% agarose gels stained with ethidium bromide using FisherBiotech electrophoresis system (Fisher Scientific, Pittsburgh, PA).

2.5 Real time RT-PCR

The *Bos taurus* primer pairs used for quantitative real time PCR are given in Table I. Primer pairs for CYP1A1, 1A2, 2C9, 2C19 and 3A5 genes have been described previously (Giantin et al., 2008). The primers for the remaining genes and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to the sequences in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) with the help of Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Specificity of the primers used was confirmed by BLAST analysis of the GenBank database from NCBI (Edgar et al., 2002).

One microliter of the reverse transcription product (cDNA) was added to the PCR mixture containing 5 μL of SYBR[®] Advantage[®] premix, and 1 μM each of the sense and antisense primers (Table I) in a final volume of 10 μL prepared with RNase free water in the wells of the MicroAmp[®] Fast optical 48-well reaction plate maintained on ice. The wells were covered with the thermal seal adhesive film, and the plate was centrifuged at $250 \times g$ for 30 seconds at 4°C using an Eppendorf 5804R centrifuge (Eppendorf, Hauppauge, NY). The real time PCR reactions were performed in a StepOne PCR machine (Version 1.0, Applied Biosystems[®], Life Technologies[™], Carlsbad, CA). The PCR run was initiated with a 5-minute cycle at 95°C . A three step cycling program was used to monitor amplification for

40 cycles with a 30-second denaturing step at 95 °C, a 30-second annealing step at a primer-specific annealing temperature (Table I) and a 30-second elongation step at 72 °C. At the end of the PCR cycle, a melting (dissociation) curve analysis was performed to verify the amplification of a single amplicon. Negative control reactions with no added reverse transcriptase product were performed for each PCR run to monitor potential contamination of reagents. Electrophoretic analysis of the PCR products was performed by separating the products on ethidium bromide-stained 2 % agarose gels and visualizing by transillumination with ultraviolet light. The real time PCR products were purified using the QIAquick® gel extraction kit and sequenced by the DNA Sequencing Facility of the University of Iowa (Iowa City, IA).

2.6 Calculation of gene expression levels and statistical analysis

For real time PCR, the relative expression level (L) for each gene was calculated from the threshold cycle value (C_T (Schmittgen and Livak, 2008)) using the equation $L = 2^{-C_T}$. Normalization of the expression level of the CYP genes to the endogenous control (GAPDH) was performed by dividing the expression levels of each gene of interest by the expression level of GAPDH. The tissue expression of mRNA for each CYP enzyme was expressed as mean $\pm$ standard deviation of six samples from different animals. Statistically significant differences between expression levels of CYP enzymes among the nasal olfactory and respiratory mucosae and the liver were evaluated using one-way ANOVA and pairwise comparisons were performed using Student's t-test with a Bonferroni correction. A p-value of less than 0.05 was considered to be significant.

2.7 Histological examination of bovine nasal mucosa

Fixed nasal mucosal explants were dehydrated and embedded in low temperature melting paraffin using an RMC paraffin tissue processor (Model 1530, Kroslok Enterprises Inc., Midland, ON, Canada). The slides for histological examination were prepared at the Central Microscopy Research Facility at the University of Iowa (Iowa City, IA). For morphologic examination, 5 μ m thick sections were cut using a Leica UC6 ultramicrotome (Leica Microsystems Inc., Bannockburn, IL) and mounted onto Superfrost® Plus microscope slides (Fisher Scientific Inc., Pittsburgh, PA). The mounted sections were dehydrated overnight and deparaffinized by placing them in an oven maintained at 60 °C to melt the paraffin followed by rinsing the slides in xylene. Staining of the sections with hematoxylin and eosin was carried out using a DRS-601 Sakura Diversified Stainer (GMI Inc., Ramsey, MN). Finally, the sections were coverslipped and examined using an Olympus BX-51 light microscope (Olympus America Inc., Center Valley, PA) equipped with a DP-71 digital camera.

2.8 Immunohistochemistry

The slides for immunohistochemical examination were prepared at the Central Microscopy Research Facility at the University of Iowa (Iowa City, IA). For CYP immunofluorescence imaging, 5 μ m sections of respiratory and olfactory mucosa were deparaffinized and dehydrated using a DRS-601 Sakura Diversified Stainer (GMI Inc, Ramsey, MN). Heat-induced epitope retrieval of the sections immersed in citrate buffer was carried out in a microwave (Pelco-Biowave, Ted-Pella Inc, Redding, CA) set at 650 watts with a target temperature of 95 °C (5 min warm, 5 min rest, 5 min warm). Non-specific binding was

minimized by incubating the sections in blocking buffer (10% normal goat serum, 1% bovine serum albumin in phosphate buffered saline (PBS), pH 7.4) for 20 minutes at room temperature. The sections were incubated with the following dilutions of primary antibody solutions for 90 minutes at room temperature: mouse anti-human CYP1A2 (1:100), mouse anti-human CYP2A6 (1:1000), rabbit anti-human CYP2C (1:1000) and rabbit anti-human CYP3A4 (1:1000). The primary antibody aliquots were diluted to the desired concentrations in PBS. Negative controls were incubated with species-matched, non-specific immunoglobulins at concentrations identical to those of the primary antibodies.

After rinsing in PBS, the tissues were exposed to secondary antibody solutions [goat anti-mouse Alexa 488 (1:500) for CYP1A2 and CYP2A6 or goat anti-rabbit Alexa 488 (1:500) for CYP2C and CYP3A4] for 30 minutes at room temperature. Following washing, the sections were incubated with the nuclear stain, To-Pro3, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). A BioRad 1024 confocal laser-scanning microscope (BioRad, Carl Zeiss, Germany) equipped with a krypton/argon laser at excitation wavelengths of 488 nm and 647 nm was used to examine the section. Digital image processing was performed using ImageJ software (Abramoff et al., 2004; Schneider et al., 2012) (U.S. National Institutes of Health, Bethesda, MD). For comparison of the signal intensity of each CYP enzyme between the olfactory and respiratory mucosae, the nasal explants were matched for animal donor, antibody concentration and the staining protocol, and the incubations were all performed at the same time.

3. Results

The data obtained by RT-PCR confirmed the expression of several CYP genes in the bovine olfactory and respiratory mucosae. As seen in gel electrophoresis images (Figure 1), transcripts of each of the enzymes selected were detected as real time PCR products. Figure 2 shows the gene expression levels of the selected CYP enzymes normalized with respect to GAPDH. Bovine hepatic tissue exhibited significantly higher gene expression levels for all the CYP enzymes examined (CYP1A1, CYP2A6, CYP2C31, CYP2C42, CYP3A4, and CYP3A5), than the corresponding mRNA levels in the olfactory and respiratory mucosa. No statistically significant differences were observed between the mRNA expression levels obtained from the olfactory and the respiratory mucosa for any of the genes studied. Both of the nasal tissues exhibited abundant expression of three genes: CYP2A6, CYP3A4 and CYP3A5. The remaining enzymes, including CYP1A1, CYP2C31, and CYP2C42 were expressed in both bovine olfactory and respiratory mucosa, but exhibited low expression levels compared to those obtained in bovine hepatic tissues (Table II). The RT-PCR results from the current studies showed that the expression of the CYP2C42 mRNA was mainly confined to bovine hepatic tissues. The expression levels of CYP2C31 mRNA obtained in both bovine olfactory and respiratory tissues were significantly higher than those obtained for CYP2C42 mRNA in the bovine olfactory and respiratory mucosa.

Amplification of the CYP1A2 gene from mRNA extracted from bovine respiratory mucosal tissues resulted in multiple PCR products, yielding multiple bands on electrophoretic analysis as shown in Figure 3. Figure 4 demonstrates the observed gene expression levels of CYP1A2 in bovine hepatic, olfactory and respiratory tissues. Real time PCR monitoring of

one out of six olfactory tissues and four out of six respiratory tissues resulted in an indeterminate C_T value for the desired transcript of CYP1A2. To identify the sequences of the fragments amplified during real time RT-PCR, the individual DNA fragments were extracted from the agarose gel and sequenced to identify possible sequence homology with the bovine CYP1A2 genes (NCBI accession number NM_001099364.1). The sequence of the amplified products partially aligned with the nucleotide sequence of bovine CYP1A2 mRNA, indicating the possible expression of a spliced variant of CYP1A2 genes in the bovine olfactory and respiratory mucosae.

The histological organization of the bovine olfactory mucosa, obtained from the ethmoid turbinate is shown in Figure 5. The olfactory mucosa consists of a pseudostratified columnar epithelial layer and an underlying highly-cellular lamina propria. The submucosal region contains a significant population of the cells of Bowman's glands and blood vessels. The respiratory mucosa, shown in Figure 6, has a similar cellular organization, where the pseudostratified columnar epithelial layer is interspersed with mucus producing goblet cells, and the lamina propria contains respiratory serous glands, blood vessels and connective tissue.

Due to the possibility of undesirable cross-reactivity among different bovine CYP epitopes with commercially-available primary antibodies, only one enzyme from each of the CYP subfamilies (1A, 2A, 2C, and 3A) was selected for the investigation of representative protein localization in bovine nasal mucosa. Cross-reactivity between different enzymes of the CYP subfamily could not be ruled out during these studies, however, the overall expression patterns obtained for the enzymes studies were identical, confirming CYP distribution in specific tissues of the bovine nasal olfactory and respiratory mucosa.

Immunohistochemical analyses (Figures 7 and 8) of the localization of the CYP enzymes showed that the enzymes belonging to the subfamilies 1A, 2A, 2C, and 3A were expressed in the bovine nasal mucosa where positive immunoreactivity is indicated by green fluorescence. ToPro3 was used to stain the nuclei red, which helped distinguish the cytoplasmic green fluorescence signal obtained from the cellular immunostaining from that of the autofluorescent red blood cells. In the olfactory mucosal sections, CYP 3A4, 2C8/9/19, and 2A6 protein immunoreactivity was observed in the pseudostratified columnar epithelial layer and the acinar cells of Bowman's glands in the submucosal layer (Table II). In the immunopositive tissues, immunofluorescence was mainly confined to the cytoplasm. Submucosal endothelial cells did not exhibit significant immunoreactivity. Replacement of the primary antibody with species-matched immunoglobulins (negative control) in the staining protocol resulted in a markedly lower level of staining in the tissue sections, including the endothelial cells which are a common site for nonspecific IgG staining. For the respiratory mucosal sections, strong immunoreactivity was observed in the pseudostratified columnar epithelium and the serous glands for all four CYP subfamilies studied, including 3A, 2C, 2A, and 1A. This, as previously explained, could be due to cross-reactivity of primary antibodies towards multiple CYP enzymes. While the CYP1A2 fluorescent signal obtained in the respiratory sections was stronger than that obtained for the olfactory sections (Figures 7 and 8), the signal obtained in the olfactory sections treated for CYP1A2

immunofluorescence was not visibly different from the signal obtained in the negative control.

4. Discussion

Gene expression levels examined by RT-PCR illustrated that CYP2A6, CYP3A4, and CYP3A5 were the dominant CYP enzymes in the bovine olfactory and respiratory mucosal tissues, and a summary of the comparison of reported CYP expression in rats, human and bovine hepatic and nasal tissues is shown in Table II. The prominent expression of CYP2A6 in the bovine nasal mucosa was anticipated, as the enzymes of the CYP2A subfamily have been shown to be mainly extra-hepatic, and their nasal mucosal expression and activity has been extensively studied in humans and laboratory animals (Chen, 2003; Gu et al., 2000; Su and Ding, 2004; Su et al., 2000, 1996). Su et al. demonstrated that the CYP2A subfamily was present in human and rat nasal mucosa, but the relative mRNA expression levels in humans were much lower than the expression of the CYP2A genes in the rodent nasal mucosa when normalized with respect to each species' liver CYP2A content (Su et al., 1996).

The enzymes of the CYP3A subfamily play key roles in catalyzing hepatic first-pass metabolism of orally administered therapeutic agents. CYP3A enzyme expression and activity has been extensively studied in the hepatic tissues of humans and rodents (Meredith et al., 2003; Nishimuta et al., 2013; Watanabe et al., 2004), and studies on cattle liver expression have also been reported (Maté et al., 2015). However, information available about the presence and activity of CYP3A enzymes in the nasal mucosa is scarce. CYP3A proteins have been previously identified in the respiratory and olfactory mucosa of rats, rabbits and humans by immunohistochemistry (Ding and Kaminsky, 2003; Thornton-Manning, 1997; Yokose et al., 1999), and CYP3A4-like gene expression was reported by Darwish et al. in mRNA samples extracted from bovine lungs and kidneys (Darwish et al., 2010(b)). Quantitative analysis of nasal mucosal CYP3A expression has not been previously reported, however. Real time RT-PCR with bovine primer pairs targeted towards two CYP3A enzymes showed that both bovine CYP3A4 and CYP3A5 mRNAs were expressed in the bovine olfactory and respiratory mucosae of the 6 animals studied. The expression levels were approximately 1–12% of the corresponding gene expression levels in the liver. The expression of several members of the CYP3A subfamily in bovine olfactory and respiratory tissues suggests that the bovine nasal mucosa possesses significant levels of CYP3A4-like catalytic activity.

CYP1A1 was expressed in both the bovine olfactory and respiratory mucosa, but exhibited low expression levels (< 1%) compared to bovine hepatic tissues. CYP1A1 is primarily an extra-hepatic enzyme, as its hepatic expression is reported to be very low in rats and humans (Hadley, 1982; Ling et al., 2004; Martignoni et al., 2006; Schweikl et al., 1993; Voigt et al., 1993). However, our results demonstrating the expression of CYP1A1 in bovine liver are in agreement with previous reports where CYP1A1 has been shown to be highly expressed in the hepatic tissues in cows (Darwish et al., 2010 (a); Sivapathasundaram et al., 2001). CYP1A is also the most inducible CYP subfamily, and many chemicals present in dietary plant components, in addition to environmental pollutants like polycyclic aromatic

hydrocarbons have been reported to enhance its expression (Ioannides, 1999; Ling et al., 2004). Given the typical pasture characteristics for domestic livestock in the midwestern United States, dietary or environmental CYP1A1 inducers may have played a major role in influencing the apparent expression of CYP1A1 mRNA in the bovine liver samples that were used in these studies.

For the case of CYP1A2 mRNA amplification by RT-PCR, the liver and the nasal tissues presented notable differences in mRNA expression patterns among the six animals studied. The mRNA extracted from the livers of all six animals exhibited nearly identical expression of the CYP1A2. However, amplification of the CYP1A2 transcript from mRNA extracted from some of the bovine olfactory and respiratory mucosal tissues yielded multiple products. Real time PCR monitoring of one of six olfactory tissues and four of six respiratory tissues resulted in indeterminate C_T values for the desired transcript of CYP1A2 (Figure 4). The variability in bovine CYP1A2 nasal expression is not entirely surprising, as pronounced inter-individual differences (40–130 fold) have been reported in CYP1A2 activity in humans (Gunes and Dahl, 2008; Zhou et al., 2010). A significant amount of work has been directed towards studying the genetic, epigenetic and environmental factors affecting CYP1A2 expression and catalytic activity in humans and rodents (Eaton et al., 1995; Gunes and Dahl, 2008; Ikeya et al., 1989; Rajkumar et al., 2013; Zhou et al., 2009).

The CYP2C subfamily primarily consists of 2C8, 2C9, 2C18, and 2C19 enzymes in humans. The enzymes of this subfamily constitute approximately 20% of total human hepatic CYP contents (Shimada et al., 1994), and the expression of CYP2C mRNAs and proteins has been reported in the small intestine and other extra-hepatic tissues in humans (Klose et al., 1999). In rats, the CYP2C subfamily is abundantly expressed in the hepatic tissues and is involved in the oxidation of chemicals like aflatoxin B1 and steroids (Martignoni et al., 2006; Nedelcheva and Gut, 1994).

The enzyme distribution patterns observed from the immunohistochemical analyses indicate inter-species similarity in nasal CYP enzyme distribution, and the epithelial and submucosal enzyme distribution patterns are consistent with the reported cellular localization of CYP in the human nasal mucosa (Getchell et al., 1993) as well as the distribution of CYP2A related proteins in fetal and adult human nasal mucosa (Chen, 2003) and with the staining patterns obtained for CYP2S1 in human nasal mucosa by in situ hybridization and immunohistochemistry (Saarikoski et al., 2005). Similar observations were also reported by Voigt et al. who observed CYP1A1 immunoreactivity localized in the cells of Bowman's glands in rat olfactory mucosa with minimal staining of the epithelium (Voigt et al., 1993). Our findings are also consistent with the observed cytochrome P450 localization in epithelia, sustentacular cells and Bowman's glands in rat olfactory mucosa (Chen et al., 1992).

5. Conclusions

Bovine nasal tissues possess a multi-cellular organization comparable to the adult human nasal mucosa, and results from the RT-PCR and immunohistochemical studies demonstrate that bovine nasal olfactory and respiratory mucosae express similar populations and distributions of CYP enzymes as have been previously reported in other species, including

humans and rats. The expression and localization patterns of cytochrome P450 enzymes in the bovine nasal mucosa makes these tissues a promising *ex vivo* tool for pharmacotoxicological studies in the investigation of enzyme-induced transformations of chemicals by the nasal mucosa, including potential toxicities from environmental exposures or diminished effectiveness of therapeutic agents targeted to or absorbed via the nasal mucosa.

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Abbreviations

cDNA	complementary DNA
DNA	Deoxyribonucleic acid
CYP	Cytochrome P450
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCl	Hydrochloric acid or hydrochloride
IgG	Immunoglobulin G
KCl	Potassium chloride
MgCl₂	Magnesium chloride
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction

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Figure 1.

Gel electrophoretic analysis of RT-PCR products obtained during amplification of the selected CYP genes from bovine hepatic, olfactory, and respiratory tissues extracted from six different cows.

Figure 2.

Gene expression levels of the selected CYP enzymes normalized with respect to GAPDH expression in bovine hepatic, olfactory, and respiratory tissues analyzed using real time RT-PCR. Results are presented as mean $\pm$ standard deviation for 6 animals. Error bars represent standard deviations. * represents statistically significant difference in the enzyme expression levels for liver vs. the nasal (olfactory or respiratory) mucosa at $p < 0.05$. No statistically significant differences between the mRNA expression levels were observed between the olfactory and the respiratory mucosa for any of the enzymes studied.

Figure 3.

Gel electrophoretic analysis of RT-PCR products from amplification of CYP1A2 gene in bovine hepatic, olfactory, and respiratory tissues. Amplification of the CYP1A2 gene from mRNA extracted from several bovine respiratory and olfactory mucosal tissues resulted in multiple PCR products. Lanes showing multiple bands following amplification are highlighted with ovals.

Figure 4. Gene expression levels of the CYP1A2-like gene normalized with respect to GAPDH expression in bovine hepatic, olfactory, and respiratory tissues analyzed using real time RT-PCR. Real time PCR monitoring of one of six olfactory tissues and four of six respiratory tissues resulted in an indeterminate C_T value for the desired transcript of CYP1A2.

Figure 5. Brightfield image of a formalin-fixed, paraffin-embedded, and hematoxylin and eosin stained section (5 μm) of bovine olfactory mucosa. Scale bar: 200 μm.

Figure 6. Brightfield image of a formalin-fixed, paraffin-embedded, and hematoxylin and eosin stained section (5 μm) of bovine respiratory mucosa. Scale bar: 200 μm.

Figure 7. Immunohistochemical staining of CYP1A2 (A), CYP2A6 (B–C), CYP2C (D), and CYP3A4 (E) in bovine olfactory mucosa. A representative negative control image is shown in F. Green fluorescence indicates positive immunoreactivity; red coloration indicates nuclei (To-Pro3). Scale bar: 20 μm.

Figure 8. Immunohistochemical staining of CYP1A2 (A), CYP2A6 (B–C), CYP2C (D), and CYP3A4 (E) in bovine respiratory mucosa. A representative negative control image is shown in F. Green fluorescence indicates positive immunoreactivity; red coloration indicates nuclei (To-Pro3). Scale bar: 20 μ m.

Table I

Primers used for quantitative real time RT-PCR (Edgar et al., 2002)

Enzyme	Primers	Sequence (5'-3')	Product size (bp)	Annealing temp (°C)	Position (5')	Accession number
Bovine CYP1A1	Sense	GACCTGAATCAGAGGTTCTACGTCT	81	60	861	<u>XM_588298.7</u>
	Antisense	CCGGATGTGACCCCTCTCAA			941	
Bovine CYP1A2	Sense	ACCATGACCCGAAGCTGTG	78	60	1301	<u>NM_001099364.1</u>
	Antisense	CAATGGTGGTGCCATCAGAC			1378	
Bovine CYP2A6	Sense	CCAGATGGAGCTCTTCCTCT	195	58	26	<u>DQ114539.1</u>
	Antisense	CTATTGTCTCTCCCAACCAG			220	
Bovine CYP2C42	Sense	TCCCTGGACATGAACAACCC	71	60	760	<u>XM_612374.5</u>
	Antisense	TTGTGCTTTTCTGTTCATCTT			830	
Bovine CYP2C31	Sense	TCCCAAGGGCACAAACCATA	56	60	1146	<u>XM_600421.5</u>
	Antisense	CCTTGCCATCGTGCAGG			1201	
Bovine CYP3A5	Sense	GCCAGAGCCCGAGGAGTT	77	60	1309	<u>NM_174531.2</u>
	Antisense	GCAGGTAGACGTAAAGGATTTATGCT			1385	
Bovine CYP3A4	Sense	GGAAACCTGGGTTCTCCTGGCT	308	58	114	<u>NM_001099367.1</u>
	Antisense	CCGATGGACCAAAAACCCCTCG			421	
Bovine GAPDH	Sense	ATGGAGAAGGCTGGGGCTCACT	209	58	379	<u>NM_001034034.2</u>
	Antisense	AGTCCCTCCACGATGCCAAAGT			587	

Comparison of reported CYP expression in human, rat, and bovine hepatic and nasal tissues

Enzyme	Human liver	Human nasal mucosa	Rat liver	Rat nasal mucosa	Bovine liver	Bovine nasal (olfactory and respiratory) mucosa
CYP1A1	mRNA: Low Protein: Low and variable (Martignoni et al., 2006; Schweikl et al., 1993)	mRNA: moderate (Ling et al., 2004)	mRNA: not detected Protein: low (Martignoni et al., 2006)	mRNA: moderate (Minn et al., 2005)	mRNA: moderate	mRNA: moderate
CYP1A2	mRNA: variable Protein: variable (Gunes and Dahl, 2008; Schweikl et al., 1993; Zhou et al., 2010)	mRNA: moderate and variable (Ling et al., 2004; Zhou et al., 2010)	mRNA: moderate-to-high (Lu and Li, 2000)	mRNA: moderate (Minn et al., 2005)	mRNA: high	mRNA: variable Protein: weak
CYP2A Subfamily	mRNA: high (Martignoni et al., 2006; Koskela et al., 1999)	mRNA: low in adults, high CYP 2A mRNA in fetal olfactory mucosa (Gu et al., 2000; Koskela et al., 1999; Su et al., 1996)	mRNA: low (Martignoni et al., 2006)	mRNA: moderate (Robottom-Ferreira et al., 2003; Su et al., 1996)	mRNA: moderate (CYP2A6)	mRNA: moderate Protein: high (CYP2A6)
CYP2C Subfamily	mRNA: high Protein: high (Shimada et al., 1994)	Protein: moderate (Yokose et al., 1999)	mRNA: high Protein: high (Nedelcheva and Gut, 1994; Martignoni et al., 2006)	mRNA: low (Minn et al., 2005)	mRNA: high	mRNA: low Protein: high
CYP3A Subfamily	mRNA: high Protein: high (Watanabe et al., 2004; Shimada et al., 1994)	Protein: moderate (Yokose et al., 1999)	mRNA: high and variable Protein: high and variable (Martignoni et al., 2006; Meredith et al., 2003; Nishimuta et al., 2013)	mRNA: moderate Protein: moderate (Minn et al., 2005; Thornton-Manning, 1997)	mRNA: high (Maté et al., 2015)	mRNA: high Protein: high

Scoring of enzyme expression is based on our RT-PCR and immunohistochemistry results for bovine nasal and hepatic mucosa and an extensive qualitative review of available literature for nasal cytochrome P450 gene and protein expression in humans and rats. For bovine hepatic RT-PCR expression, a normalized expression of greater than 1000 units (expression level in hepatic tissues/GAPDH expression in hepatic tissues) was assigned a "high" score, and a normalized expression of 40–1000 units was assigned a "moderate" score. For bovine nasal RT-PCR expression, a normalized expression of greater than 150 units (expression level in nasal tissues/GAPDH expression in nasal tissues) was assigned a "high score"; a normalized expression of 5–15 units was assigned a "moderate score"; a normalized expression of <5 units was assigned a "low" score.