Investigations of Sodium Dependent Vitamin C Transporters in the Ovine Corpus Luteum

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Introduction

Ascorbic acid, the reduced form of Vitamin C, plays a role in several of the body's essential biological functions. Within the cell, ascorbic acid scavenges for free radicals that pose a threat to the organism's well being. Also, collagen synthesis cannot be completed without the role of vitamin C as co-factor in its enzymatic reactions (Friedman and Zeidel, 1999, Hediger, 2002). Ascorbate also functions as a reducing agent for metal ions within the body (Rubin et al., 2005). While most mammals are able to synthesize ascorbic acid from glucose, primates and guinea pigs lack the physiological capabilities to do so, therefore all Vitamin C must be obtained from the diet (Wilson, 2005).

With all of the different functions ascorbate performs within the body, transport in and out of the cell in an efficient manner is absolutely essential for ascorbate to perform its duties intracellularly. Ascorbate is transported into the cell with the help of either Sodium Dependent Vitamin C Transport Protein 1 or 2 (SVCT1 or 2), depending on the absorption location within the body. SVCT1 is found primarily in the epithelial cells of the intestine and kidney, and found intracellularly in the apical cell membrane. The gene Slc23a1 codes for the synthesis of SVCT1 (Wilson, 2005). Although SVCT1 plays an important role in the transport of ascorbate across the cell membrane, it is not expressed as widely in bodily tissues as SVCT2. SVCT2 is found in tissues such as the brain, placenta, eye, and exocrine and endocrine tissues. Within the cell SVCT2 is found concentrated within the basolateral cell membrane, and it is encoded for by the gene Slc23a2 (Rubin et al., 2005, Wilson, 2005).

Both transport proteins require two sodium ions in order to transport the ascorbic acid across the cell membrane. The electrochemical gradient created by the sodium ions provides the transport protein with the energy needed for translocation (Wilson, 2005). Both transport proteins will bind one sodium ion initially, then an ascorbic acid molecule and, lastly, the second sodium ion will be bound. Both sodium ions and the ascorbic acid are transported across the cell membrane at the same time (Mackenzie et al., 2007). If sodium is not available to the SVCT proteins, then translocation of ascorbic

acid can occur via binding of either Mg^{2+} or Ca^{2+} (Godoy et al., 2006).

Although SVCT1 and 2 are the only functional forms of a sodium dependent transport protein for Vitamin C, a non-functional version of hSVCT1 has been found to exist in Caco2 cell lines. The non-functional transport protein is 92% homologous with the functional SVCT1 protein, although it contains a four amino acid insertion which, when deleted, results in a fully functional SVCT1 protein (Wang et al., 1999). Interestingly, a non-functional truncated version of the human SVCT2 protein has also been reported. This truncated hSVCT2 protein contains a 345 bp deletion without a frame shift. While this protein is not functional, it is a potent negative inhibitor of both SVCT1 and 2, although it has a stronger effect on SVCT2. It is hypothesized that the truncated form of hSVCT2 inhibits the carrier proteins through protein interaction (Lutsenko et al., 2004).

The corpus luteum (CL) is a tissue where the function of SVCT proteins is of interest. The CL plays a crucial role in the maintenance of pregnancy and also the return of the animal to estrus. Should fertilization occur within the animal, the corpus luteum will continue to secrete the hormone progesterone. If fertilization does not occur, the CL regresses and the animal will return to its estrous cycle (Stocco et al., 2007).

The corpus luteum is formed in response to a cascade of events, beginning with the release of luteinizing hormone (LH) from the anterior pituitary gland in a pre-ovulatory surge. This surge of LH causes ovulation of the follicle in the ovary and also begins the process of luteinization within the follicle cells. Specifically, the LH surge causes the granulosa cells of the functional follicle to leave the cell cycle so that they can be transformed through luteinization. The thecal and granulosa cells within the follicle will be turned into small and large steroidogenic luteal cells (Stocco, et al., 2007). The main function of the luteal cells is to make progesterone from cholesterol. The primary source of cholesterol for this purpose is circulating low density lipoproteins. The entire lipoprotein is engulfed and taken into the cell, and after a series of enzyme-catalyzed reactions, the cholesterol is released from the

lipoprotein and migrates to the mitochondria. Within the mitochondria, the cholesterol side chains are cleaved by the enzyme P450scc and turned into pregnenolone. Pregnenolone is then transported out of the mitochondria and converted to progesterone. Estradiol and androgens are also produced within the luteal cells (Stocco, et al., 2007) to varying degrees depending on the species.

Should pregnancy not occur, the CL will regress. The process of regression of the corpus luteum is referred to as luteolysis. Luteolysis can be considered to have two components: structural and functional regression. While the structural regression refers to the physical aspect of luteolysis, functional regression is characterized by the inhibition of progesterone production by $PGF_{2\alpha}$ (Stocco, et al., 2007). $PGF_{2\alpha}$ is released from the uterus in pulses, which creates a positive feedback on the CL to produce $PGF_{2\alpha}$ itself, although the CL seems to only be responsive to $PGF_{2\alpha}$ in the mid to late luteal phase. Research shows that, when injected with $PGF_{2\alpha}$, a corpus luteum with luteolytic capacity will produce more $PGF_{2\alpha}$-- evidence of the positive feedback loop (Wiltbank and Ottobre, 2003). The importance of luteolysis, as stated above, is to allow estrus to begin again and LH to be released from the anterior pituitary gland. (Stocco et al., 2007).

Many events occur within the corpus luteum during its short existence, including a raging battle between antioxidants, such as ascorbate, and reactive oxygen species (ROS). Reactive oxygen species tend to accumulate in the CL, because they are produced as an end product of steroidogenesis. Macrophages and neutrophils present in the CL also produce ROS. As levels of $PGF_{2\alpha}$ rise within the corpus luteum and it starts to regress, two specific types of reactive oxygen species, superoxide radicals and hydrogen peroxides, increase within the corpus luteum. A decrease in the amount of blood flowing to the ovaries during luteolysis also contributes to the increase in the amount of ROS within the corpus luteum. These two ROS have also been shown to inhibit the production of progesterone within the corpus luteum in both rat and human cell cultures. ROS produced during steroidogenesis also impede antioxidants' ability to scavenge (Sugino, 2006). The enzyme superoxide dimutase (SOD), another

antioxidant, shows increased expression in the bovine estrous cycle from days 6-16. The concentration of SOD rapidly decreases as the progesterone made by the corpus luteum decreases (Rapoport et al., 1998). Similar to SOD, ascorbate concentrations also decreased along with progesterone (Petroff et al., 1998).

In studies performed on porcine corpora lutea, researchers found that ascorbate concentrations were highest within the corpus luteum during the mid to late luteal phase, the period of maximum function (Petroff et al., 1997). The lowest ascorbate concentrations were found to be during days 1-3 of the estrous cycle, when the CL is developing, and days 13-16 of the estrous cycle, when the CL is regressing. During one study, porcine corpora lutea were injected with $PGF_{2\alpha}$ in vivo, and then, using blood samples from both peripheral and utero-ovarian veins, ascorbate concentrations were monitored every fifteen minutes for two hours post injection. Two hours after $PGF_{2\alpha}$ injections, ascorbate concentrations within the CL had decreased. Ascorbate concentrations had increased in blood plasma collected from the utero-ovarian vein during the same time period. Utero-ovarian plasma concentrations were elevated as compared to the blood plasma from the ear vein, suggesting that ascorbate was exiting the corpus luteum in response to the increased levels of $PGF_{2\alpha}$ (Petroff et al., 1998). In a study along the same lines, rat corpora lutea cell cultures were treated with C^{14} labeled ascorbic acid. When injected with $PGF_{2\alpha}$, the uptake of ascorbate into the CL was inhibited. When LH and progesterone were injected into the cells there was no effect on ascorbate intake (Musicki et al., 1996). According to unpublished data (Gaddis and Ottobre, personal communication), injection of sheep with $PGF_{2\alpha}$ during the early luteal phase caused a decrease in luteal ascorbate concentrations two hours post injection, however the concentrations recovered after twenty-four hours. When the same procedure was repeated in the mid luteal phase, ascorbate concentrations decreased after two hours, but did not recover. These data suggest that ascorbate within the corpus luteum is especially sensitive to $PGF_{2\alpha}$ in the mid luteal phase.

As stated previously, the ascorbate concentrations within the corpus luteum follow the same trend as progesterone concentrations within the CL. However, ascorbate seems to have another relationship with progesterone. In a study using JEG-3 and JAR cell lines, treatment of the cells with ascorbic acid actually stimulated the production of progesterone and estradiol. The mRNA expression of enzymes responsible for steroidogenesis were also stimulated by treatment with ascorbic acid. To test these findings, SVCT mRNA was knocked down within the cell lines, which means that no ascorbic acid could be transported across the cell membranes. Without any ascorbic acid transport, there was a decrease in the expression of the enzymes responsible for steroidogenesis. Treatment of the cells with another antioxidant, α-tocopherol, did not produce any of the same results. Therefore, ascorbic acid could play a vital role in scavenging ROS that inhibit progesterone synthesis. In addition, ascorbate could also be helping to maintain the life of the CL through progesterone and estradiol production (Wu et al., 2008).

Should fertilization occur within the animal, there is an increase in the amount of Cu and Zn SOD enzymes within the corpus luteum. The hormone human chorionic gonadotropin (hCG), seems to be responsible for the increase in expression of SOD enzymes. This could suggest that during pregnancy in humans the corpus luteum is able to scavenge ROS much more efficiently than during luteal regression. This increase in scavenging abilities could contribute to the increase in the life span of the corpus luteum, which is essential for a successful pregnancy (Sugino, 2006).

The SVCT1 sequences from the following species have been identified according to BLAST at the NCBI server: human, mouse, rat, pig, and guinea pig. The human sequence has 598 amino acids with twelve membrane domains, while the rat sequence has 694 amino acids (Eck et al., 2004, Wang et al., 1999, and Tsukaguchi et al., 1999). SVCT2 was sequenced second, and has been identified in the human, mouse, rat, pig, guinea pig, dog, and rabbit, according to BLAST. The rat SVCT2 sequence contains 592 amino acids, and is 65% homologous to SVCT1 (Tsukaguchi et al., 1999).

While there has been much research on the mouse, rat, and human SVCT protein sequences, the ovine sequence has only been partially identified. Currently 296 base pairs of the ovine SVCT1 protein, which translate into 98 amino acids, have been sequenced. SVCT2 has 1860 base pairs and 618 amino acids sequenced (Ceddia, 2005).

Objectives

One of the main objectives of this experiment was to see if the sheep had a truncated version of SVCT2. This isoform has been previously noted in the human and is a potent inhibitor of SVCT1 and 2 (Lutsenko et al.,2004). Previous research had sequenced part of SVCT 1 and 2, however neither the 3' or 5' end of either protein had been sequenced. This project aimed to sequence the 3' end of both proteins.

Experimental Design

PCR primer design and reactions:

Ovine corpora lutea were obtained on day ten of the estrous cycle during a previous study (information from unpublished data from Ottobre and Gaddis). RNA from the CL was previously isolated using RNAqueous-4PCR Kit (Ambion Inc., Austin, TX). The total RNA concentration collected was 821.5 ng/µL, but was diluted to a concentration of 78ng/µL for use in PCR reactions (concentrations estimates from Nanodrop). PCR primers were previously designed with the aid of the Primer3 program (S. Rozen and H.J. Skaletsky, 2000) and made by Invitrogen (Carlsbad, CA).

Primers designed to determine the existence of a short isoform of oSVCT2 were created based on the partially sequenced oSVCT2. The primers, named primer set 1, 5'GGATAGCACTGGCAGTCTAGACC 3' (forward) and 5' GCCATACTTCGTGCTGTCAGGAG 3' (reverse) were designed to be both upstream and downstream from the predicted deleted sequence. If the deleted sequence was present in the sheep CL, then an amplicon of 463 bp would be observed along with the expected 804 bp amplicon from the complete (untruncated) oSVCT2 message. Cycling

conditions were based on the Superscript 1-step RT-PCR with Platinum Taq protocol sheet from Invitrogen (Carlsbad, CA). Two controls were put in place for the PCR. One negative control did not contain RT/Platinum Taq Mix, but did contain two units of DNA polymerase. This control was put in place to see of the CL RNA had been contaminated with any genomic DNA. The other negative control that was designed did not contain any CL RNA to test if there was any other RNA contamination in the sample. The PCR program consisted of 30 minutes at 50° C, 2 minutes at 94° C and 36 cycles of 15 seconds at 94°C, 30 seconds at 57°C, 1 minute at 72°C. 57°C was used because optimum annealing temp is at least 4°C less than Tm of the primers. The final extension was 1 cycle of 72°C for 7 minutes.

Primer Sets 11&12 were designed to determine the 3' end of SVCT1. The expected length was determined to be 2182 bp based on the bovine SVCT1 mRNA sequence. Primer set 11 forward was designed to be 5'ACTGGGGTATCTCAGCGCT3'. Primer set 12 forward was 5'TGGGGTATCTCAGCTTGC3'. Primer sets 11&12 were run with the same PCR conditions as primer sets 5&6 (see below). Primer sets 5&6 were designed to determine the 3' end of SVCT2. The expected length was determined to be 2173 bp based on the bovine SVCT2 mRNA sequence. Primer Set 5 forward was 5'CGCTAGTAGCCGGAGTTCAGAT3', and Primer Set 6 forward was 5'CTACTTACCCATCAGCCCAACCT3'. Both primers were designed based on the partially sequenced oSVCT2. Because it was the 3' end being sequenced, the reverse primer for all 3' end primer sets was Oligo dT 3' primer provided by Invitrogen Gene Racer Superscript III RT Rxn Kit (Carlsbad, CA). The CL RNA was reverse transcribed using Oligo dT primers based on the procedure suggested by the Superscript III RT Rxn Kit. Five controls were put in place for amplifying both the SVCT1&2 3' ends. The positive control tube contained primer set 1 forward and reverse. This was used to check the RT-PCR quality since an amplification of 804 bp had already been successfully accomplished (Paice and Ottobre, preliminary data). One of the negative controls also contained primer set 1 forward and reverse, but no template was added to determine the presence of DNA contamination. The second

negative control did not contain any of the forward primer sets to see if there was any non specific binding of the Oligo dT 3' primer. The last two negative controls did not contain any Oligo dT 3' primer, but did contain one of the four 5' end forward primers. This control was put in place to see if there was any non specific binding of the gene specific primers.

The cycling conditions for the SVCT1&2 3' ends were 1 cycle of 94°C for 2 minutes, 6 cycles of 94°C for 30 seconds, 72°C for 2 minutes and 12 seconds, 6 cycles of 94°C for 30 seconds, 70°C for 2 minutes and 12 seconds, 23 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 2 minutes and 12 seconds. The final cycle was 68°C for 10 minutes. All cycling parameters were based on suggestions by the Invitrogen Gene Racer Superscript III RT Rxn Kit.

Gel Electrophoresis and Staining:

For all gel electrophoresis procedures, a 1% agarose minigel Bio-Rad Ready Gel was run on a Bio-Rad Mini-Sub Cell GT electrophoresis system (Hercules, CA). For primer set 1 forward and reverse a 100 bp DNA ladder (Invitrogen, Carlsbad, CA) was used, and 2µL Bio-Rad nucleic acid sample buffer was used as dye (Hercules, CA). The electrophoresis system ran at 30mA for 40 minutes, at which time the blue stain was at the bottom of the gel. For primer sets 11, 12, 5&6 a 1000 bp DNA ladder (Invitrogen, Carlsbad, CA) was used, and 2µL Bio-Rad nucleic acid sample buffer as dye (Hercules, CA). The electrophoresis system ran at 30mA for 90 minutes. All gels were stained using a SYBR Gold stain, and rocked on Boekel Rocker II for 25 mins each. The gels were viewed under a UV light to observe the bands.

Results

Gel electrophoresis staining of the first PCR reaction was designed to investigate the presence of a truncated version of SVCT2 in the sheep CL. A single band was visualized in the second and third lanes of the gel (Figure 1). Both lanes contained our experimental reaction with the sheep CL RNA and forward and reverse primers from primer set 1. The reaction was run in duplicate to ensure accuracy.

When compared against the DNA ladder, the bands in each lane were approximately 800 bp in length. No other bands were visualized on the gel in any of the control lanes.

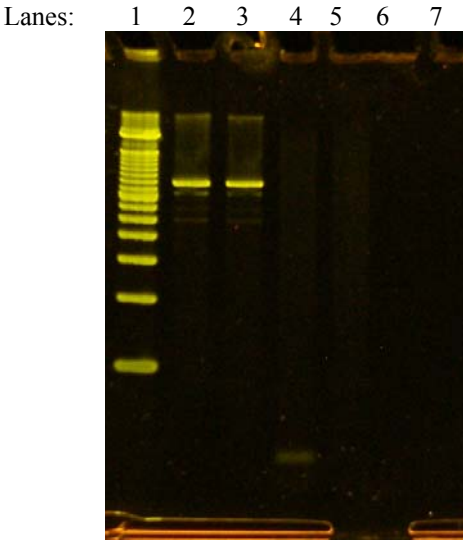

Figure 1. Lane 1 contained 100 bp DNA ladder (Invitrogen, Carlsbad, CA). Lanes 2&3 contained Sheep CL RNA and Primer Set 1 Forward & Reverse. Lanes 4&5 contained a negative control, which did not contain RT/Platinum Taq Mix. Lanes 6&7 contained a negative control, which did not have any sheep CL RNA.

The gel that resulted from the PCR run of primer sets 5 and 6 showed a band in the second and third lanes (Figure 2). The second lane was a positive control containing primer set 1 forward and reverse. A band of approximately 800 base pairs was visualized, as was expected if the RT-PCR was successful. The other band was visualized in the lane representing the reaction containing primer set 5 forward and Oligo dT 3' primer, and was approximately 1800 bp in length.

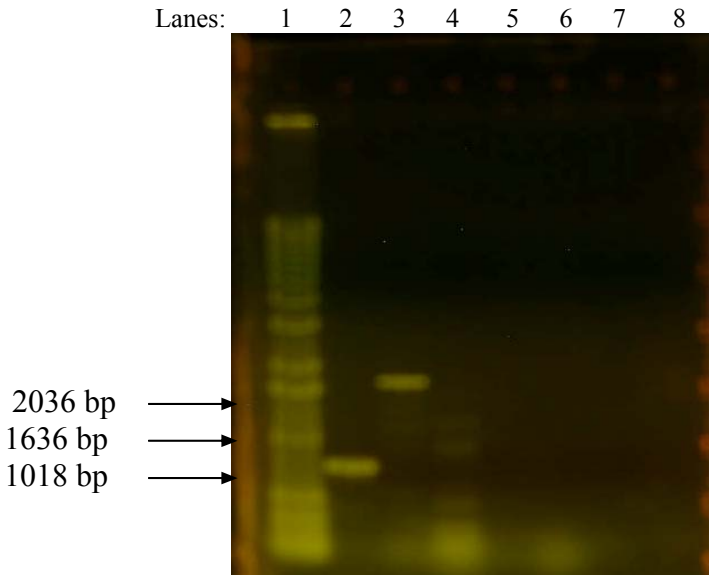

Figure 2. Lane 1 contained 1000 bp DNA ladder (Invitrogen, Carlsbad, CA). Lane 2 contained a positive control, which had primer set 1 forward & reverse. Lane 3 contained primer set 5 forward and Oligo dT primer 3'. Lane 4 contained primer set 6 forward and Oligo dT primer 3'. Lane 5 contained a negative control, which contained primer set 1 forward & reverse but not template. Lane 6 contained a negative control, which did not contain any forward primer sets. Lane 7 contained a negative control, which contained primer set 5 forward but no Oligo dT primer 3'. Lane 8 contained a negative control, which contained primer set 6 forward but no Oligo dT primer 3'.

Gel electrophoresis from primer sets 11 and 12 again yielded a band for the positive control containing primer set 1 forward and reverse (Figure 3). No other bands were visualized, however.

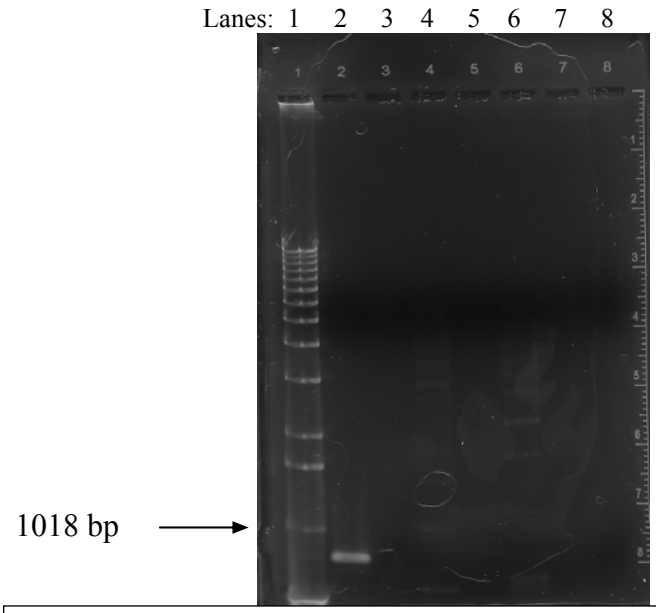

Figure 3. Lane 1 contained 1000 bp DNA ladder (Invitrogen, Carlsbad, CA). Lane 2 contained a positive control, which had primer set 1 forward & reverse. Lane 3 contained primer set 11 forward and Oligo dT primer 3'. Lane 4 contained primer set 6 forward and Oligo dT primer 3'. Lane 5 contained a negative control, which contained primer set 1 forward & reverse but not template. Lane 6 contained a negative control, which did not contain any forward primer sets. Lane 7 contained a negative control, which contained primer set 5 forward but no Oligo dT primer 3'. Lane 8 contained a negative control, which contained primer set 6 forward but no Oligo dT primer 3'.

Discussion

Because the band from the primer set 1 experimental reaction yielded an 800 base pair band, but not a 463 bp band, we have no evidence to suggest that the sheep contains a shortened version of SVCT2, which has been found to exist in the human (Lutsenko et al., 2004). This region of the SVCT2 message has already been sequenced (Ceddia, 2005), and we used primers that would yield an 800 bp amplicon based on this known sequence. The primers used flanked the homologous region in the human SVCT2 message where there is a deletion in a truncated and inhibitory isoform. If the sheep had a similar isoform, we anticipated observing a 463 bp band in addition to the 800 bp band. This finding

is promising, because if the sheep does not have this inhibitory version of SVCT2, sheep may be better equipped to absorb vitamin C within the CL than if they had this inhibitor. Because the 800 bp band showed up so nicely, it was used as a positive control for both primer set 11&12 and also 5&6. The band appeared on both gels, furthering the proof that there is no truncated version of oSVCT2.

Primer set 5 yielded one band other than the positive control. This band was approximately 1800 bp. This band was a little shorter, but somewhat similar to the expected length based on the bovine sequence (i.e., 2173 bp). DNA sequencing is needed to determine the nucleotide sequence of the 3' end of oSVCT2. Interestingly, primer set 6 did not yield any bands, but this was a different primer designed by the Primer3 program. More PCR reactions could be run to see if different cycling parameters could yield results. None of the negative controls yielded any results, suggesting that there was no DNA contamination or non-specific binding of the Oligo dT 3' primer or gene specific primers.

Unfortunately, there were no bands visualized from the experimental reactions of primer sets 11 and 12. The positive control containing primer set 1 resulted in the expected band of 800 bp, suggesting that the RT-PCR quality was good. In addition, no bands appeared in negative control lanes. Changing the conditions of the PCR reactions may be important to optimize the initial binding of these two sets of primers.

Conclusion

There is strong evidence that the sheep does not contain a truncated version of SCVT2 as observed in the human. There is also evidence that the 3' end of SVCT2 is approximately 1800 base pairs, however further sequencing is needed.

The results from this research bring the research community one step closer to sequencing and understanding both ascorbic acid transport proteins. Once the entire sequence is known, there will be more flexibility in primer design, and real time PCR can be used to investigate the regulation of these important transporters. As such, researchers can gain a better understanding of the regulation of ovarian

ascorbate, increase our knowledge of the role of ascorbate in reproduction, and utilize this information to better manipulate and enhance reproduction in sheep.

Works Cited

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