Alkaline phosphatase expression in tissues from glucocorticoid-treated dogs

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**Objective**—To determine the effect of glucocorticoids on the induction of alkaline phosphatase (ALP) isoenzymes in the liver, kidneys, and intestinal mucosa, 3 tissues that are principally responsible for ALP synthesis in dogs.

**Sample Population**—Tissues from the liver, kidneys, and intestinal mucosa of 6 dogs treated with 1 mg of prednisone/kg/d for 32 days and 6 untreated control dogs.

**Procedures**—Using canine-specific primers for the ALP isoenzymes, a reverse transcription-polymerase chain reaction assay was designed to measure liver ALP (LALP) and intestinal ALP (IALP) mRNA and heterogeneous nuclear RNA (hnRNA) expression in tissues from the liver and kidneys and intestinal mucosa of glucocorticoid-treated and control dogs. Tissue ALP isoenzyme activities were compared between the groups.

**Results**—The LALP activity and mRNA concentrations increased in tissues of the liver and kidneys in dogs treated with prednisone, whereas LALP hnRNA increased only in liver tissues. The IALP activity and mRNA expression increased in intestinal mucosa and liver tissues in prednisone-treated dogs. We did not detect an increase in IALP hnRNA expression in these tissues.

**Conclusions and Clinical Relevance**—Synthesis of ALP is increased in the liver, kidneys, and intestinal mucosa of dogs in response to prednisone treatment. This response appears to be regulated at the transcriptional level, but mechanisms may differ between LALP and IALP. (Am J Vet Res 2002;63:1083–1088)

Alkaline phosphatase (ALP) activity in healthy dogs is found primarily in tissues obtained from the intestines, kidneys, liver, and bones, with intestines and kidneys having the greatest activity per gram of tissue.1,2 The ALP isoenzymes are named for their tissue of origin. There are 3 ALP isoenzymes in serum: liver ALP (LALP), corticosteroid-induced ALP (CIALP), and bone ALP (BALP).1,2,3,4 By far, LALP has the greatest activity in serum of clinically normal dogs, and LALP activity can increase with cholestatic hepatic diseases as well as with glucocorticoid excess.5 Serum BALP activity is greatest in young growing animals or animals with pathologic conditions of osseous tissue, such as neoplasia.6 Serum BALP activity increases minimally with excess glucocorticoids.7

Corticosteroid-induced ALP is a unique isoenzyme of dogs, and most dogs have little or no CIALP activity in serum.1,4,5 In dogs, increased serum or tissue ALP activity is associated with excess circulating glucocorticoids.7,9 Under conditions of excess circulating glucocorticoids, ALP activity, in particular CIALP activity, increases significantly in the serum and liver.1 It is likely that increased tissue and serum ALP isoenzyme activity is controlled at the level of gene transcription under the influence of glucocorticoids. However, we are not aware of any studies that have examined the expression of ALP isoenzymes in various canine tissues under the influence of glucocorticoids, and in general, there is little known about the mechanisms underlying ALP expression in dogs.

Two genes control ALP in dogs.10,11 The first gene is the tissue nonspecific (TNS) ALP gene, which produces LALP, BALP, and kidney ALP isoforms. These 3 principle isoforms of the ALP TNS gene differ only in degree of glycosylation.10,11 The second gene, the intestinal ALP (IALP) gene, is specific for the IALP isoenzyme product and is believed to be expressed only in the intestinal mucosa of dogs.10 The term isoform refers to similar forms of an enzyme transcribed from the same gene but that are altered differently during the posttranslational events. The term isoenzyme refers to similar enzymes that perform the same catalytic reaction but are from different genes. Enzyme kinetics, inhibition studies, antigenic properties, and N-terminal amino acid sequencing have confirmed that CIALP is a product of the IALP gene.10-12 Intestinal ALP and CIALP differ only in carbohydrate composition.9 Because CIALP activity increases in hepatic tissue of glucocorticoid-treated dogs, it is likely that IALP is also expressed in this tissue.

Measurement of mRNA transcripts in a particular tissue provides an indirect assessment of gene transcription. The accumulation of mRNA represents the sum of transcriptional and posttranscriptional events. Therefore, increased mRNA transcripts and increased transcription are not synonymous. To measure the rate of transcription in a particular tissue, other techniques must be used in addition to those that measure mRNA transcripts alone.

A technique to measure the rate of transcription of a particular gene is a reverse transcription-polymerase chain reaction (RT-PCR) assay of heterogeneous nuclear RNA (hnRNA).13,14 Heterogeneous nuclear RNA are unspliced transcripts of mature mRNA and represent a subpopulation of total RNA contained within a cell. The amount of unspliced hnRNA is a function of the rate of transcription and processing within a cell.15 Measurement of hnRNA by use of RT-PCR follows the same principle as measurement of mature mRNA tran-
scripts by use of RT-PCR assays. The major difference for the RT-PCR assay designed for measurement of hnRNA is the primer pair used. For amplification of hnRNA, it is essential that at least 1 primer be nested within an intervening intron. Introns are only found within unspliced mature mRNA and genomic DNA; therefore, these are the only templates that are recognized by the intron-nested primer. Because the intron-nested primer cannot discriminate between genomic DNA and hnRNA, rigorous methods must be used to avoid the inclusion of contaminating genomic DNA. There are several variations of the RT-PCR assay for use in measurement of hnRNA, but all adhere to the same fundamental goal of amplifying unspliced hnRNA.

Because of the unique characteristics and clinical importance of ALP isoenzymes in dogs, the study reported here was performed to gain a basic understanding of the effect of glucocorticoids on the induction and expression of ALP isoenzymes in canine epithelial cells. Expression of ALP isoenzymes in other epithelial cell populations was examined to gain further insight into ALP expression in dogs in response to glucocorticoids.

**Materials and Methods**

Animals—Twelve mixed-breed male dogs were used in the study. All dogs were housed at the Laboratory Animal Facility at our institution. Use of the dogs was approved by the University of Illinois Laboratory Animal Care Advisory Committee.

Procedure—Six dogs received injections of prednisone (1.0 mg/kg SC, q 24 h) for 32 consecutive days. On day 32, the dogs were euthanatized by administration of barbiturate overdose, and tissues were harvested and stored in various conditions. From each dog, samples (approx 1 to 2 g) of the liver, kidneys, and intestinal mucosa of the duodenum were harvested, snap frozen, and stored in liquid nitrogen for RNA extraction, whereas additional samples (approx 20 to 50 g) of the liver, kidneys, and intestinal mucosa of the duodenum were harvested and stored at −20 C for assay of ALP activity. The other 6 dogs were used as control dogs in other studies. After completion of those studies, these dogs also were euthanatized. Samples of the liver, kidneys, and intestinal mucosa were subjected to treatment in the same manner as tissues from the treatment group.

RNA extraction—Total cellular RNA was extracted from tissues of the liver, kidneys, and intestinal mucosa of the duodenum that had been snap frozen and stored in liquid nitrogen. Extraction was accomplished by use of a guanidine thiocyanate-phenol-chloroform technique.

RT-PCR assay—Aliquots (1 µg) of total RNA from the liver, kidneys, and intestinal mucosa of the duodenum were reverse transcribed into cDNA by use of a commercial kit' performed in accordance with the manufacturer’s protocol. The RNA samples were treated with DNase to remove detectable amounts of contaminating genomic DNA prior to the reverse transcription reaction. Random hexamers supplied with the kit were used as the primer for reverse transcription. The resulting cDNA was diluted 1:2 with sterile, RNase-free water and used as a template for the PCR procedure.

Relative quantitation of ALP isoenzyme mRNA—Relative quantitation of mRNA transcripts in the tissues was performed by use of a commercially available multiplex PCR kit. Multiplex PCR is defined as the use of 2 or more primer sets in a single PCR procedure. One primer pair recognized the target of interest (ie, LALP and IALP); primer pairs were designed on the basis of the published canine-specific cDNA. The other primer pair was used to amplify an invariant endogenous control sample (18S rRNA). Amplification of 18S rRNA acts as a control sample for adjustment of the relative quantitation of the target gene.

Briefly, 1 µg of total RNA from each tissue sample was treated with DNase and subjected to reverse transcription, as described previously. Each PCR procedure contained 1× reaction buffer (50 mM KCl, 20 mM tris-HCl [pH 8.4], 0.1% Triton X-100), 1.75 mM MgCl2, 0.2 mM of each dinitoside triphosphate, 1 µM of the ALP-specific primers, 1 µM of the 18S rRNA-specific primers, 1.25 units of Taq DNA polymerase, and 5 µl of diluted tissue-specific cDNA template.

Sterile water replaced cDNA template and served as a negative-control sample. The PCR assay for each tissue was performed in duplicate. The thermocycle pattern included an initial denaturing step at 95 C for 3 minutes, then 35 cycles of denaturing at 95 C for 30 seconds, primer annealing at 60 C for 30 seconds, and primer extension at 72 C for 45 seconds. The final step was primer extension at 72 C for 7 minutes following by an end step at 4 C.

After completion of the PCR procedure, 10 µl of the reaction product was separated on a 1.5% agarose gel. Gels were stained with ethidium bromide in tris borate EDTA (TBE) buffer and electrophoresed at 90 V for approximately 1 hour. Resulting bands evident on the gels were quantitated by use of densitometry.

Relative quantitation of ALP isoenzyme hnRNA—Measurement of hnRNA by use of RT-PCR assay requires at least 1 PCR primer nested within an intervening intron. For this PCR procedure, a downstream primer was designed by use of ALP isoenzyme sequence data obtained from within an intron (ie, nested primer). By using this newly designed downstream primer and the aforementioned upstream primer, hnRNA could be amplified. A disadvantage of the use of a primer that is nested within an intron is the indiscriminate amplification of contaminating genomic DNA. To ensure that contaminating genomic DNA was not available as a template for the PCR assay, the RNA samples were treated with DNase prior to the RT-PCR procedure.

Two control experiments were performed to ensure that the PCR amplification product was not a result of contaminating genomic DNA. First, aliquots of total RNA from the liver and intestinal mucosa were subjected to treatment with 3 nucleases (RNase, DNase, or a combination of RNase and DNase). Treatment with RNase alone should have removed all intact RNA, thereby leaving genomic DNA for amplification by the PCR assay. Treatment with DNase alone should have removed all genomic DNA from the total RNA sample, thereby leaving the hnRNA intact for amplification by the PCR assay. Treatment with the combination of RNase and DNase should have removed all nucleic acids in the sample, and a product should not have been detectable after the PCR assay. By examining the resulting PCR products for each nuclease treatment, it can be determined whether contaminating genomic DNA was adequately removed from the RNA sample, which ensured amplification of hnRNA only.

For the RNase reaction, aliquots (1.0 µg) of total RNA from liver and intestinal mucosa were mixed with 2.0 µl of RNase, and a final volume of 10.0 µl was achieved by the addition of sterile water. For the DNase reaction, aliquots (1.0 µg) of total RNA were mixed with 1.0 µl of DNase buffer and 2.0 µl of DNase, and a final volume of 10.0 µl was achieved by the addition of sterile water. Finally, the combined RNase and DNase reaction was accomplished by use of aliquots (1.0 µg) of total RNA mixed with 1.0 µl of DNase buffer, 2.0 µl of...
DNase, and 2.0 µl of RNase; and a final volume of 10.0 µl was achieved by the addition of sterile water. All nuclease reactions were incubated at 37 C for 25 minutes.

Following incubation, reactions were terminated by the addition of 1.0 µl of 25 mM EDTA; samples and EDTA were mixed, and the solutions were incubated at 65 C for 10 minutes. The treated total RNA was then subjected to the RT-PCR procedure, using primers designed to amplify hnRNA. Amplification products were separated on a 1.5% agarose gel. Gels were stained with ethidium bromide in TBE buffer and electrophoresed at 90 V for approximately 1 hour.

The second control experiment was used to determine whether DNase treatment of total RNA prior to reverse transcription was sufficient to eliminate contaminating DNA. Following DNase treatment of total RNA from the liver and intestinal mucosa, 2 samples of the treated RNA were subjected to reverse transcription as described previously. In 1 sample, reverse transcriptase was added (RT+), whereas sterile water replaced reverse transcriptase in the other sample (RT–). All other reagents for reverse transcription were used as previously described; in addition, the PCR procedure for hnRNA was performed. Resulting PCR products were separated on a 1.5% agarose gel. Gels were stained with ethidium bromide in TBE buffer and electrophoresed at 90 V for approximately 1 hour. If DNase treatment of total RNA was successful, an amplification product would not be evident in the RT– sample, whereas the amplification product for the RT+ sample should represent hnRNA. This reverse transcription control experiment was performed 3 times on various RNA samples to ensure repeatable results. Once the hnRNA was amplified by the PCR assay, the resulting product was cloned into a commercial plasmid and sequenced to determine whether it had the desired hnRNA sequence.

The cDNA generated from reverse transcription of the tissue total RNA was used as the template for determination of TNS LALP and IALP hnRNA. An aliquot (5 µl) of cDNA from each tissue was analyzed for hnRNA by use of the PCR assay. All protocols used for measurement of mRNA transcripts in the tissue samples by use of relative quantitative RT-PCR were the same as those used for hnRNA measurement. The difference between measurement of mRNA and hnRNA was in the number of cycles necessary to amplify a hnRNA product, lack of 18S primers in the hnRNA PCR procedure, and the primers used. The PCR product for TNS LALP and IALP hnRNA was amplified for 42 cycles, rather than the 35 cycles used for mRNA.

Measurement of TNS LALP and IALP activities in liver tissues—Samples of tissue were weighed and placed in 4 volumes of 0.05M acetate buffer (pH 4.9). The tissue was homogenized in acetate buffer, using a pestle designed for use in the microcentrifuge tubes. One volume of n-butanol was added to the solution. The mixture was vortexed at 10-minute intervals for 1 hour. The mixture then was centrifuged at 16,000 X g for 2 minutes, and the supernatant was removed. The supernatant was diluted 1:10, and undiluted and 1:10, and TNS LALP and IALP activities were measured. The TNS LALP activity was measured by use of a standard p-nitrophenylphosphatase assay on an automated serum chemistry analyzer. To determine IALP activity, an inhibitory concentration of levamisole (4.2 mM) was added to the p-nitrophenylphosphatase reagent, and the extract was assayed in the same manner as for TNS LALP activity.

Statistical analysis—Mean values for all variables were compared between treated and control dogs by use of a 1-tailed Student t test. Significance was set at P < 0.05 for all tests.

Results

The TNS LALP activity in tissues of the liver and kidneys from glucocorticoid-treated dogs was significantly increased, compared with activity in tissues from control dogs (Fig 1). A significant increase in TNS LALP activity in intestinal mucosa was not observed. Relative quantitative multiplex RT-PCR assay of TNS LALP revealed a band of the appropriate size of 1,150 base pairs (bp) in addition to the 500-bp
product for the 18S control sample (data not shown). Expression of TNS LALP mRNA in tissues from the liver and kidneys of glucocorticoid-treated dogs was significantly increased, compared with expression in tissues of control dogs, as measured by use of the RT-PCR procedure (Fig 2). Expression of TNS LALP mRNA was not detectable in intestinal mucosa when tested by use of the RT-PCR procedure.

A 316-bp PCR product for TNS LALP hnRNA was detected from tissues of the liver and kidneys of control and glucocorticoid-treated dogs. The rate of TNS LALP transcription, as measured by use of the RT-PCR procedure for TNS LALP hnRNA expression, revealed a significant increase in liver tissue but an increase that was not significant in tissue from the kidneys (Fig 3). Expression of TNS LALP hnRNA was not detectable in total RNA from the intestinal mucosa.

The IALP activity in glucocorticoid-treated dogs was significantly increased, compared with activity for control dogs, in liver and intestinal tissues, whereas tissues from the kidneys had negligible IALP activity (Fig 4). The relative quantitative multiplex RT-PCR assay for IALP mRNA expression in the tissues revealed a target band of 590 bp, whereas there was a 500-bp band for the 18S control sample (Fig 5). The product of IALP expression was observed for liver tissue from the glucocorticoid-treated dogs but not the control dogs. Expression of IALP mRNA measured by RT-PCR assay in liver and intestinal mucosa tissues of glucocorticoid-treated dogs was significantly increased, compared with expression for control dogs (Fig 6). Expression of IALP mRNA was not detected in tissues of the kidneys from glucocorticoid-treated or control dogs.

A 442-bp PCR product for IALP hnRNA was detected from intestinal mucosa total RNA of control and glucocorticoid-treated dogs (data not shown). A PCR product for IALP hnRNA was observed in liver tissue obtained from 3 control and 2 glucocorticoid-treated dogs (data not shown). Transcriptional rate of IALP in the liver of glucocorticoid-treated dogs was...
increased, but not significantly, compared with values for control dogs (Fig 7). We did not detect a difference in transcriptional rate of IALP in intestinal mucosa between glucocorticoid-treated and control dogs.

**Discussion**

In the study reported here, the expression of ALP isoenzymes in dogs treated with glucocorticoids was determined for several types of epithelial cells. Prednisone treatment increased ALP isoenzyme activity and mRNA expression in the liver, kidneys, and intestinal mucosa of dogs, suggesting that the effect of glucocorticoids on ALP synthesis of epithelial cells is not limited to hepatocytes. However, the magnitude of increase in response to glucocorticoids differed among tissues. Interestingly, it appears that the effect of glucocorticoids on IALP expression in the liver of dogs was greater than the effect seen in the intestinal mucosa. A similar observation was made regarding TNS LALP in tissues of the liver and kidneys in which the observed increase in TNS LALP activity and expression in the liver of glucocorticoid-treated dogs was greater than in the kidneys. These findings are likely attributable to differences in the baseline expression of each gene in each tissue. For example, the liver has relatively low baseline TNS LALP activity and virtually nonexistent IALP expression and activity. In contrast, the kidneys and intestines normally have high baseline TNS LALP and IALP activity, respectively (ie, relatively high constitutive expression). Therefore, an increase in ALP expression in the liver appears more profound in comparison to increases for the kidneys and intestines because of low initial baseline values in the liver.

In this study, indirect measurement of transcriptional rates for TNS LALP and IALP was determined by use of RT-PCR assay of hnRNA. Measurement of hnRNA by use of quantitative RT-PCR procedures allows for only a relative assessment of transcription rate. Nevertheless, the use of this technique allowed us to assess relative differences in transcriptional rates of steady-state concentrations of hnRNA between glucocorticoid-treated and control dogs. Better quantitative techniques, such as nuclear run-on assays, were not performed because of several disadvantages for those techniques, including the need for large amounts of radioactive material, the need for large numbers of cells to obtain a sufficient number of nuclei, and problems of decreased viability of the nuclei extracted from tissues.

In the study reported here, we also compared patterns of hepatic expression for the TNS gene and the IALP gene under the influence of glucocorticoids. Hepatic TNS LALP activity, expression, and transcription rate in glucocorticoid-treated dogs were all significantly increased, compared with values for control dogs. This suggested that glucocorticoids act at the level of gene transcription and result in an increase in hepatic and serum TNS LALP activities. For hepatic IALP, there was a significant increase in mRNA expression and activity in the glucocorticoid-treated dogs, compared with values for the control dogs. However, we did not detect a significant increase in rate of IALP gene transcription. These findings suggested that glucocorticoids may not act at the level of gene transcription for induction of hepatic IALP. Differences in steady-state concentrations of mRNA that cannot be fully accounted for by changes in the rate of transcription may give an indication of posttranscriptional gene regulation. Glucocorticoids can induce posttranscriptional modifications in many systems. Studies that directly link glucocorticoids to posttranscriptional modifications of ALP expression are lacking. On the basis of these findings, it is believed that hepatic TNS
LALP responds differently to glucocorticoids than does hepatic IALP. Interestingly, tissues from the kidneys and intestinal mucosa had increased TNS LALP and IALP activity and expression, respectively, but did not have a significant increase in the rate of gene transcription. On the basis of these data, it is suspected that posttranscriptional mechanisms may be responsible for tissue expression of TNS LALP and IALP. The mechanism of such posttranscriptional events is currently unknown; however, elucidation of these mechanisms may provide additional clues to the nature of the unique patterns of expression of ALP isoenzymes in blood of dogs. Findings of the study reported here should allow future studies to focus on the nature of these posttranscriptional events.

References