SUPPLEMENTAL MATERIALS

Effects of Insulin on the Metabolic Control of Hepatic Gluconeogenesis in vivo

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SUPPLEMENTAL RESEARCH DESIGN AND METHODS

RNA extraction, cDNA synthesis and real-time PCR procedures. Total RNA was extracted by homogenizing 50 mg of canine liver in 1 mL of Tri-reagent (Sigma, St. Louis, MO) following the manufacturer's instructions. RNA was further purified using the Qiagen RNEasy kit (Qiagen, Valencia, CA). RNA purity was verified based on A260/A280 ratios greater than 1.8, and RNA integrity was verified using ethidium bromide-stained agarose gels. First strand cDNA was synthesized from total RNA using the High Capacity reverse transcription kit (ABI, Foster City, CA) as per manufacturer's directions and cDNA was stored at -20^{o}C until use. Primers were designed and analyzed using the Primer3 and mfold programs (BioRad, Hercules, CA). Real-time PCR primer specificity was determined by BLAST analysis. Primer pairs are listed in Supplemental Table 1. Primer efficiencies were validated to be between 91-96% for each primer pair, and primer specificity was confirmed by both melt curve analysis and 1% agarose gel electrophoresis (each of which revealed one product). Real-time PCR was performed using a BioRad iCycler Detection System with SYBR green fluorophore. Reactions were initiated by adding 0.25 μg of cDNA template to 50 μL total reaction mix. The PCR protocol included denaturation at 95^{o}C for 3 min followed by 40 cycles of amplification (95^{o}C for 30 s, anneal at 56^{o}C for 30 s, elongate at 72^{o}C for 30 s). Melt curve analysis was performed after every run and samples were run in duplicate. Expression of test genes were normalized relative to the housekeeping gene hypoxanthine phosphoribosyl transferase 1 (HPRT1) using the Livak method as previously described (1). Initial experiments validated HPRT1 as a housekeeping gene relative to cyclophilin. Analysis of data was performed using iCycler iQ Optical System Software Version 5.0 (BioRad).

SDS-PAGE and Western blotting procedures. Soluble protein from frozen canine liver was extracted, subjected to electrophoresis, and transferred to nitrocellulose, essentially as described by Ramnanan et al. (2). Nitrocellulose membranes, SDS-PAGE and wet-transfer reagents were supplied by Invitrogen (Carlsbad, CA). Cellular fractionation (to prepare nuclear- and cytoplasmic-enriched fractions to assay FOXO-1 localization) was performed as per Morris et al. (3). Immuno detection was performed as described (2). Primary and secondary antibodies were supplied by Cell Signaling (Danvers, MA), with the exception of sheep anti-PEPCK (gift from Dr. D.K. Granner, Vanderbilt University Medical Center), sheep anti-GK (gift from Dr. M. Shiota, Vanderbilt University Medical Center), and donkey anti-sheep secondary antibody (Sigma). Incubation times with primary and secondary antibodies, and antibody dilutions, were optimized for each test protein. Proteins were visualized using ECL Plus Western detection reagents (GE Healthcare, Piscataway, NJ, USA) following manufacturer's protocols and ECL signals were detected after exposure to BioMax Light X-ray films (Kodak, Chalon-sur-Saone,

France). Initial experiments validated that immunoreactive proteins were within the linear range of ECL detection. Protein band densities were quantified using ImageJ software (http://rsb.info.nih.gov/ij/). Test protein bands were normalized for lane-to-lane variation in loading using actin as previously described (2).

Fructose-2,6-bisphosphate ($F2,6P_2$) assay. Liver $F2,6P_2$ was extracted as previously described (4). Briefly, 50 mg of frozen canine liver was homogenized in 1 mL of 50 mM NaOH. Extracts were heated for 5 minutes at 80^{o}C, cooled, and neutralized by addition of ice-cold 1 M acetic acid in the presence of 20 mM HEPES. After centrifugation at 8000 g for 10 minutes, supernatants were assayed for $F2,6P_2$ using the potato 6-phosphofructokinase-1 (PFK-1) activation method of Van Shaftingen et al. (5). Samples were incubated in buffer containing potato PFK-1 (a kind gift from Dr. Alex Lange, University of Minnesota), 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.15 mM NADH, 5 mM fructose-6-phosphate, and 1 U/mL each of the linking enzymes glycerol-3-phosphate dehydrogenase, triosephosphate isomerase, and aldolase. The reaction was then initiated by addition of 1 mM sodium pyrophosphate. Activity was measured by detecting the rate of NADH oxidation at 340 nM at 25^{o}C. Initial assays validated linearity with respect to extract volume, assay time, and substrate concentration. A standard curve was prepared assaying PFK-1 activity in the same conditions with known amounts of $F2,6P_2$, which was another gift from Dr. Lange.

Pyruvate kinase (PK) assay. Extraction and assay of PK was performed following established protocols (6; 7). Frozen canine tissue was homogenized in buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 200 mM NaCl, 50 mM NaF, 10% glycerol, and 10 mM β-mercaptoethanol; a few crystals of phenylmethylsulphonyl fluoride and 5 μL/mL each of Sigma Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails I and II were added immediately prior to homogenization. Homogenates were centrifuged 10 000 g X 20 min at 4^{o}C, and supernatants were desalted by centrifugation through G-25 Sephadex columns as done previously (6). Desalted extracts were assayed for PK activity at 25^{o}C in buffer containing 50 mM Tris-HCl, pH 7.2, 5 mM phosphoenolpyruvate, 5 mM ADP, 0.2 mM NADH, 10 mM $MgCl_2$, 100 mM KCl, and 1 U/mL of lactate dehydrogenase. Initial assays determined linearity with respect to time, substrate, and extract volume. Activity was monitored by detecting rate of NADH oxidation at 340 nM, and expressed as U/mg protein, where 1 U represents as 1 μmol pyruvate formed (or NADH oxidized) per minute, and mg protein was determined via the Biorad protein assay as done previously (2).

Supplemental Table 1. Nucleotide sequences of dog-specific primers for Real-Time PCR.

Gene	Primer	Sequence (5'-3')	Temp. (^{o}C)	Accession Number
PEPCK	Forward Reverse	AGCTTTCAATGCCCGATTTCCAGG TCAGCTCGATGCCGATCTTTGACA	57	XM_543068
G6Pase	Forward Reverse	TGAAACTTTCAGCCACATCCG GCAGGTAAAATCCAAGTGCGAA	56	XP_855553
GK	Forward Reverse	CAGAGGGGACTTTGAAATG ATGAATCCTTACCCACAATC	57	XM_846042
HPRT	Forward Reverse	AGCTTGCTGGTGAAAAGGAC TTATAGTCAAGGGCATATCC	55	L_77488
Cyclophilin	Forward Reverse	GAAGGGATTCGGTTACAAAGGTTC ATCAAACTTCTCCCCGTAGATGG	56	AF_243140

SUPPLEMENTAL REFERENCES

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