Hepatic Gene Expression in Swine Administered Dietary Aflatoxin B1

S.M. Rustemeyer[*], W.R. Lamberson[†], D.R. Ledoux[†], K. Wells[†], and *K.M. Cammack*[*]

Introduction

Distillers grains are used as an energy source in livestock feed; however, ethanol processing concentrates mycotoxins which are problematic for monogastric livestock, limiting their use in those animal industries. Mycotoxins are produced by molds on crops grown in stressful conditions. Aflatoxins are some of the most potent mycotoxins, of which aflatoxin B1 (AFB1) is the most toxic (Diaz 2005). Feeding aflatoxin-contaminated feeds, such as ethanol by-products, can cause aflatoxicosis in livestock, which is manifest through liver damage and results in poor health and performance. No treatment for aflatoxicosis currently exists, and the best prevention is frequent and stringent testing of feedstuffs to limit consumption. Regulatory limits for AFB1 in swine feed are < 20 ppb AFB1 for young, immature pigs, < 100 ppb AFB1 for breeding pigs, and < 200 ppb AFB1 for finishing pigs (USDA (2005); FDA (2009)). Identification of molecular mechanisms and pathways altered by dietary aflatoxins may lead to improved diagnostic techniques, and development of treatment and prevention strategies for aflatoxicosis. The objective of this study was to identify hepatic genes differentially expressed in barrows fed a diet containing 0, 250, or 500 ppb AFB1 for a 70 d period.

Material and methods

Animal Trial. All animal procedures used were approved by the University of Wyoming Institutional Animal Care and Use Committee. Duroc x Yorkshire crossbred barrows (n = 90; age = 35 ± 5 d; initial BW = 14.2 ± 3.0 kg) were randomly assigned to one of nine dietary treatments in a 3 x 3 factorial design: 0 (CON), 250 (LOW) or 500 (HIGH) ppb AFB1 for 7, 28, or 70 d. Barrows were allowed a 10 d adjustment period before starting their respective dietary treatment (d -10 to d -1). Barrows had *ad libitum* access to feed and water, and were fed a standard starter ration from d 0 to d 20, and a standard grower ration from d 21 until the end of the trial period. Ground corn containing AFB1 culture material was added to the LOW and HIGH starter and grower rations to target dietary concentrations of 250 ppb and 500 ppb AFB1, respectively. Barrows were monitored daily for signs of aflatoxicosis, including decreased feed intake, icterus, rough coat and lethargy (Harvey, R.B., Kubena, L.F., Huff, W.E. *et al.* (1990)), and pen feed intake was measured daily. Pigs were euthanized on their last day on trial for tissue collections.

[*] Department of Animal Science, University of Wyoming, Laramie, WY, USA 82071
[†] Department of Animal Sciences, University of Missouri, Columbia, MO, USA 65211

RNA Sequencing. Liver samples from d 70 CON (n = 9), HIGH (n = 9), and LOW (n = 6) barrows were used for gene expression analysis using RNA Sequencing (RNA-Seq). The RNA-Seq technique measures RNA abundance produced from a given genomic sequence, or transcript. The number of times an mRNA is sequenced is relative to its abundance. From the liver samples, RNA was extracted using TRI-Reagent (Sigma-Aldrich, St. Louis, MO), and purified using the RNeasy protocol (Qiagen, Valencia, CA). Quality of each sample was confirmed using the Experion RNA Analysis Kit (Bio-Rad, Hercules, CA). Samples were sent to the University of Missouri's DNA Core for RNA-Seq analysis using mRNA libraries prepared according to standard DNA Core protocol for 42-base pair short reads. Three samples were included in each lane of the flowcell; barcoded adapters were used to distinguish samples. Flowcells were sequenced using the Illumina Genome Analyzer (Illumina, San Diego, CA), and transcript abundance (i.e. transcript copy number) was reported for each sample.

Statistical Analyses. For each transcript, linear regression of transcript copy number on level of treatment (CON = 0; LOW = 1; and HIGH = 2) was performed. Correlation coefficients were also estimated between transcript copy number and treatment level. Transcripts with a significant ($r \geq |0.80|$; $P < 0.01$) correlation between copy number and treatment level were annotated using available human genomic sequence. Functional theme discovery was performed with the Expression Analysis Systemic Explorer (EASE) program within the Database for Annotation, Visualization, and Integrated Discovery (DAVID; Dennis, G., Jr., Sherman, B.T., Hosack, D.A. *et al.* (2003); Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009)). A modified Fisher exact test was used within EASE to identify overrepresented functional themes. For transcripts within functional theme(s) of interest, the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) was used to estimate least squares means and test for pair-wise differences in transcript copy number using a Tukey adjustment.

Results and discussion

Performance. Pen feed intake was lower ($P < 0.01$) in HIGH and LOW barrows than CON barrows from d 29 onward, and was lower ($P < 0.05$) in HIGH barrows than LOW barrows from day 42 onward. Average daily gain was lower ($P < 0.01$) in HIGH barrows than CON barrows from d 49 onward, and was similar between CON and LOW barrows except on d 69, when ADG was less ($P < 0.05$) in LOW barrows. These reductions in feed intake and average daily gain are similar to those observed by Southern, L.L. and Clawson A.J. (1979) and Lindemann, M.D., Blodgett, D.J., Komegay, E.T. *et al.* (1993) in swine administered AFB1-contaminated feeds.

RNA-Seq. In total, 82,744 sequences were probed to estimate transcript copy number. Of those, 179 transcripts had copy numbers highly correlated ($r \geq |0.80|$; $P < 0.01$) with treatment level; 46 sequences showed a negative relationship between copy number and treatment level, and 133 showed a positive relationship. The 179 highly correlated transcripts were annotated using available human genomic databases to determine the genes those transcripts represented. Functional analysis of the genes revealed themes of apoptosis, regulation (positive and negative) of cell growth, oxidoreductase activity, protein ligase

activity, protein binding, mRNA splicing and processing, methyltransferase activity, metabolic processes, hemopoiesis, sexual reproduction, and transcriptional activity and regulation. The apoptosis category was the most highly overrepresented, with 16 genes (Table 1). Of those 16 genes, 14 had greater copy numbers in the LOW and HIGH treatment groups, indicating that these genes were up-regulated in response to the AFB1-contaminated diet; the other 2 genes were down-regulated in response to the AFB1. One down-regulated gene has a proposed role in blood coagulation (*Kininogen 1*), and the other has been implicated in prostate cancer (*Pim-1 oncogene*). The apoptosis gene with the greatest correlation coefficient (*Cyclin-dependent kinase inhibitor 1A*) is a regulator of cell cycle progression and is itself controlled by tumor supporessor protein p53. This gene was up-regulated in the AFB1-treated barrows, as was the gene coding for the p53 target zinc finger protein. Interestingly, the overexpression of that p53 target gene has been associated with inhibition of tumor cell growth (Hellborg, F., Qian, W., Mendez-Vidal, C. *et al.* (2001)).

Table 1: Apoptosis genes with treatment differences ($P < 0.05$) in transcript copy number.

Gene	CON [1,2,3]	LOW [4]	HIGH [5]
Cyclin-dependent kinase inhibitor 1A	187.62 ± 77.81[a]	1,117.23 ± 95.30[b]	1,481.85 ± 82.53[c]
p53 target zinc finger protein	6.04 ± 9.69[a]	76.72 ± 11.86[b]	129.43 ± 10.27[c]
Bcl2/Adenovirus E1b, interacting protein 2	12.22 ± 1.48[a]	22.15 ± 1.82[b]	31.24 ± 1.57[c]
Protein kinase, AMP-activated, alpha 1	19.92 ± 4.04[a]	48.93 ± 4.95[b]	64.79 ± 4.29[b]
Hypothetical protein Flj21620	44.36 ± 14.18[a]	197.09 ± 17.36[b]	221.53 ± 15.04[b]
Mitochondrial ribosomal protein S30	46.85 ± 6.67[a]	90.82 ± 8.17[b]	116.20 ± 7.07[b]
Signal transducer and activator of transcription 1	75.65 ± 17.83[a]	192.26 ± 21.84[b]	251.85 ± 18.91[b]
Protein phosphatase 2	192.69 ± 19.50[a]	309.36 ± 23.88[b]	380.87 ± 20.68[b]
Amyloid beta precursor protein binding protein 1	26.39 ± 3.33[a]	51.76 ± 4.08[b]	59.38 ± 3.53[b]
Phosducin-like 3	41.44 ± 5.13[a]	64.79 ± 6.28[b]	86.29 ± 5.44[c]
Cullin 2	76.04 ± 8.71[a]	135.22 ± 10.67[b]	159.77 ± 9.24[b]

3 NADH dehydrogenase Fe-S protein	153.80 ± 10.95[a]	212.02 ± 13.41[b]	252.01 ± 11.61[b]
FEM-1 homolog B	47.02 ± 7.94[a]	81.88 ± 9.72[b]	115.99 ± 8.42[c]
Tyrosine 3- and tryptophan 5-monooxygenase	258.67 ± 47.76[a]	540.10 ± 58.50[b]	687.45 ± 50.66[b]
Kininogen 1	589.33 ± 27.04[a]	303.43 ± 33.11[b]	292.79 ± 28.68[b]
Pim-1 oncogene	67.95 ± 4.27[a]	36.50 ± 5.23[b]	27.26 ± 4.53[b]

[1]Least squares means ± standard error;[2]Within a row, means without a superscript in common differ ($P < 0.05$);
[3]CON = 0 ppb AFB1; [4]LOW = 250 ppb AFB1; [5]HIGH = 500 ppb AFB1.

Conclusion

Results from this study demonstrate that feeding an AFB1-contaminated diet for an extended period, even at low concentrations, is detrimental to the health and performance of growing barrows. Changes in the expression of genes related to cellular stress and toxicity responses, such as apoptosis, regulation of cell growth and proliferation, and mRNA processing, are differentially regulated in response to AFB1. Further investigations of these genes may lead to opportunities for novel treatment and prevention strategies for aflatoxicosis in swine fed AFB1-contamined feedstuffs.

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