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Original Article

Time-Dependent Regulation of Apoptosis by AEN and BAX in Response to 2-Aminoanthracene Dietary Consumption

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ABSTRACT

Background/Objective: The modulation of the toxic effects of 2-aminoanthracene (2AA) on the liver by apoptosis was investigated. Fisher-344 (F344) rats were exposed to various concentrations of 2AA for 14 and 28 days. The arylamine 2AA is an aromatic hydrocarbon employed in manufacturing chemicals, dyes, inks, and it is also a curing agent in epoxy resins and polyurethanes. 2AA has been detected in tobacco smoke and cooked foods. **Methods:** Analysis of total messenger ribonucleic acid (mRNA) extracts from liver for apoptosis-related gene expression changes in apoptosis enhancing nuclease (AEN), Bcl2-associated X protein (BAX), CASP3, Jun proto-oncogene (JUN), murine double minute-2 p53 binding protein homolog (MDM2), tumor protein p53 (p53), and GAPDH genes by quantitative real-time polymerase chain reaction (qRT-PCR) was coupled with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 (Casp3) activity assays. **Results:** Specific apoptosis staining result does not seem to show significant difference between control and treated animals. This may be due to freeze-thaw artifacts observed in the liver samples. However, there appears to be a greater level of apoptosis in medium- and high-dose (MD and HD) 2AA treated animals. Analyses of apoptosis-related genes seem to show AEN and BAX as the main targets in the induction of apoptosis in response to 2AA exposure, though p53, MDM2, and JUN may play supporting roles. **Conclusion:** Dose-dependent increases in mRNA expression were observed in all genes except Casp3. BAX was very highly expressed in the HD rats belonging to the 2-week exposure group. This trend was not observed in the animals treated for 4 weeks. Instead, AEN was rather very highly expressed in the liver of the MD animals that were treated with 2AA for 28 days.

Key words: 2-aminoanthracene, apoptosis enhancing nuclease, apoptosis, Bcl2-associated X protein, F344, quantitative real-time polymerase chain reaction

INTRODUCTION

2-Aminoanthracene (2AA), also called anthramine, is a member of a class of compounds referred to as amino-substituted polycyclic aromatic hydrocarbons (PAHs). This compound is a known mutagen and carcinogen that occurs naturally. 2AA, along with other PAHs, can be found in cereals, grains, flour, vegetables, fruits, processed meat, contaminated cow's milk, and human breast milk. Incomplete combustion of fuel, pyrolysis of fossil fuels, and wood and industrial emissions are other sources of PAHs.[1,2] The Agency for Toxic Substance and Disease Registry (ATSDR) has designated anthracene along with others such as pyrene and phenanthrene as priority PAHs due to their harmful effects, which are representative of other PAHs. Moreover, these PAHs and

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associated analogs seem to be more harmful, have greater environmental concentration, and present the greatest exposure possibilities.[1]

2AA, like other arylamine compounds, typically undergoes metabolic activation in the liver primarily to exert its toxic effects. A recent report also indicated that 2AA was activated by P450 2A13 and 2A6 (as well as P450 1B1) in *Salmonella typhimurium* species.[3] This initial activation is followed by catalysis, then by N-acetyltransferases (NAT), and finally by sulfotransferases to yield highly reactive intermediates. These electrophilic reactive metabolites form deoxyribonucleic acid (DNA) adducts, thus affecting transcription and replication.[4-7]

Global gene expression patterns in the liver[2] and pancreas[8] of Fisher-344 (F344) rats' response to 2AA dietary consumption were previously examined. Differentially expressed transcripts in the pancreas showed proteins to be involved in energy metabolism and protein digestion. The rest the expressed genes revealed messenger ribonucleic acids (mRNAs) involved in pancreatitis and pancreatic cancer.[8,9] A follow-up study evaluated the differentially expressed genes in hepatic tissues in F344 rats due to 2AA toxicity. Results revealed highly expressed transcripts observed to be actively involved in such processes as DNA repair, multidrug resistance, cell-cell adhesion, growth regulator-tumor suppressor, tissue development and differentiation, cell cycle regulation, apoptosis, and tissue senescence. Further analysis via association bioinformatics tool confirmed that biological process and molecular functions related to apoptosis and apoptotic processes are important aspects of 2AA toxicity responses.[2]

Apoptosis is the term used to describe the organized disintegration of the cell. This process is characterized by membrane blebbing, cell shrinkage, and chromatin condensation. DNA fragmentation also occurs during apoptosis. Apoptosis is believed to play essential roles in biological processes such as embryonesis, ageing, and many diseases including cancer. The apoptotic process, which includes a sequence of events, commences with initiation and then moves on to gene regulation and effector mechanism. Initiators are events that deprive survival factors such as cytokines and, in the process, activate death receptors. As a consequence of these stimuli, several varied pathways associated with specific gene expression patterns can then be generated. Proteases, named caspases, are reported to be the main apoptotic effectors.[10-14]

The current investigation examines the role of apoptosis in mediating the toxicity effects of 2AA more completely. This study is a follow-up to our previous investigation that revealed apoptosis and apoptotic events as important in mediating 2AA toxicity in the liver. We report the immunohistochemical and targeted gene expression quantification responses of cellular and molecular markers of apoptosis and apoptosis-related regulatory genes in control and exposed individuals.

MATERIALS AND METHODS

Experimental design

Male F344 rats were fed a 2AA contaminated diet. Twenty-four 3-4 week post-weaning animals (Harlan Laboratories, Madison WI) were assigned into dose regimes of 0 mg/kg-diet (control - C), 50 mg/kg-diet (low dose (LD)), 75 mg/kg-diet (medium dose (MD)), and 100 mg/kg-diet (high dose (HD)) 2AA for 14 or 28 days. Each dose regimen had at least three animals. The current doses were selected based on the findings of a previous study.[15] A pilot study by Boudreau *et al*.,[15] showed that these chosen concentrations of 2AA were nonlethal after chronic administration of the test compound. The goal of the current investigation was to provide some mechanistic details on the toxicity of 2AA. That is, to examine the toxicity mechanism of the action of 2AA. The duration of exposures-14 and 28 days-though short, provides the opportunity to do that. It also enables us to attempt to replace long-term studies with short-term bioassays.

The animals were housed in individual cages at Southern Illinois University Animal Facility with a 12-h/12-h light/dark cycle. The rats had access to distilled water *ad libitum*. The animals were handled according to the guidelines from the National Institute of Health (NIH) and Southern Illinois University Guide for Care and Use of Laboratory Animals (IACUC protocol#09-039). At the end of 14 or 28 days' treatment period, the F344 rats were euthanized with CO_2 and blood was collected via cardiac puncture. The livers of the rats, together with other major organs, were excised, weighed, and immediately frozen in liquid nitrogen.

Diet preparation

The 2AA (CAS# 613-13-8) was purchased from Sigma-Aldrich (St Louis, MO, USA) at 98% purity and used without further purification. The appropriate amount of the 2AA was dissolved in 1 L molecular grade ethyl alcohol. This was followed by the immersion of 1 kg diet (PMI Nutrition International, LLC, Brentwood, MO, USA) into the ethyl alcohol containing the 2AA and evaporated under the hood with periodic mixing to ensure homogeneity. The prepared diet was stored in the freezer and protected from light until fed to the animals.

Total RNA extraction

Total RNA was extracted from the rat livers using RNeasy Mini Kit (Qiagen, Valencia, CA).[15] Approximately 30 mg liver samples were homogenized in tissue lysis buffer to

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denature and inactivate ribonucleases (RNases). The RNA was then allowed to bind to a silica-gel membrane and finally eluted with RNase-free water. Total RNA quality and concentration were determined via electrophoretic gels and Experion™ RNA StdSens analysis kit according to the manufacturer's specifications (Bio-Rad Laboratories Inc, Hercules, CA, USA).[17]

Apoptosis assay

The presence of apoptotic cells was detected with the TUNEL Apoptosis Detection biotin-labeled POD Kit from GenScript (L00297)[18] according to the manufacturer's protocol. The frozen liver samples were quickly thawed and post fixed overnight in 10% neutral buffered formalin (NBF). The samples were paraffin embedded and sectioned at 5 μm onto glass slides. Briefly, after the deparaffinization of the 5 μm formalin-fixed paraffin-embedded (FFPE) sections, the samples were washed with phosphate buffered saline (PBS) and incubated with 0.02 mg/ml proteinase K at 37°C for 20 min. Cells were blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature (RT). The sections were incubated with a TUNEL labeling mixture of terminal deoxytransferase (TdT) and biotinylatedd UTP at 37°C for 1 h, followed by two washes in PBS for 5 min each. Streptavidin-horseradish peroxidase (HRP) was bound to the biotin molecules for 30 min at 37°C and the apoptotic cells were visualized with a 3,3'-diaminobenzidine (DAB) solution. After a final wash with PBS, the slides were cover slipped and visualized. For control staining, the positive control section was treated for 15 min at 37°C with DNaseI (25 U/μl) before incubation with the TUNEL labeling mix while the negative control section was incubated in the TUNEL labeling mix without TdT.

Caspase activity via Casp3/7 gloAssays

Casp3 activity was measured in liver tissues using Caspase-Glo assay kit[19,20] and modified protocol.[21] The assay involves cleaving of the proluminescent substrate containing DEVD (sequences were in a single-letter amino acid code) by caspases-3. This is followed by the release of the substrate luciferase (aminoluciferin) that leads to luciferase reaction and the generation of luminescent signal. To assess the Casp3 activity, the liver samples were homogenized in hypotonic buffer for cytosolic extracts. The buffer contained 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM Pefablock, and 1 μg/mL each pepstatin, leupeptin, and aprotinin. The homogenized samples were then centrifuged (15 min, 13,000 rpm, 4°C) and the subsequent protein concentration of the supernatant were adjusted to 1 mg/mL using isolation buffer. The samples were stored at −80°C. Finally, a 1:1 ratio of reagents and 10 μg/mL cytosolic protein were mixed in a white-walled 96-well plate and incubated at room temperature for 1 h.[19-21] The Casp3 activity was measured in triplicate via luminescence (relative light units with blanks subtracted) using a plate-reading Synergy 2 SL luminescence microplate according to the manufacturer's guidelines (BioTeK, Winooski, VT). Significant differences in caspase activity between the control and the treated groups were evaluated using one-way analysis of variance (ANOVA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of key gene transcripts reported to be important in meditating apoptotic processes was examined via quantitative RT-PCR. Genes, whose expression levels were quantified, included apoptosis enhancing nuclease (AEN), Bcl2-associated X protein (BAX), tumor protein p53 (p53), murine double minute-2 p53 binding protein homolog (MDM2), Jun proto-oncogene (JUN), Casp3, and GAPDH as a house-keeping gene. FASTA mRNA sequences of these mRNA transcripts were obtained for *Rattus norvegicus* using the National Center for the Biotechnology Information (NCBI) database. Forward and reverse primers for the genes were then generated using NCBI Primer-Blast. The primer sequences are shown in Table 1. The primers were bought from Integrated DNA Technologies Inc (IDT), Coralville, IA, USA.

An iScript cDNA synthesis kit (Bio-Rad Laboratories Inc)[17] was employed to synthesize complementary DNAs (cDNAs) from the total RNA extract samples of the rat livers. The cDNAs were then combined with primers and SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc.) for the qPCR reaction. The product was quantified via a Bio-Rad CFX96™ instrument (Bio-Rad Laboratories Inc.) using the manufacturer's guidelines. The normalized gene expression values were determined via relative delta delta Ct quantification parameter.

RESULTS

Detection of apoptosis using TUNEL assay

The modulating effect of 2AA on apoptosis and apoptotic

Table 1: qRT-PCR primer pair sequence and related product size

Gene	Forward primer	Reverse primer	Product size
AEN	AATCGAGCCTGTTTTCCCTT	AAAGTCCTGCCTGAGCCATA	146
BAX	ACCAAGAAGCTGAGCGAGTG	CCCAGTTGAAGTTGCCATCA	157
CASP3	GAAACCTCCGTGGATTCAAA	AGCCCATTTCAGGGTAATCC	124
JUN	CTTTGTTACTGAAGCGGGGA	GGTTACTGTAGCCGTAGGCG	206
MDM2	TTTCACGTGTGCAAAGAAGC	AAGACAATGCTCCGGCTTTA	192
p53	GAAGCTCCAGTTCATTGGGA	GACTCAGAGGGAGCTCGATG	174
GAPDH	CTCTGCTCCTCCCTGTTCT	CTTGACTGTGCCGTTGAACT	246

qRT-PCR = Quantitative real-time polymerase chain reaction

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related cellular processes in the liver was examined. TUNEL universal apoptosis detection system showed apoptotic nuclei stained dark brown and nonapoptotic nuclei stained blue [Figure 1]. We think the freezing and thawing of the liver samples may have an effect on the staining of the nuclei. Nevertheless, the livers of the treated rats in both treatment groups seem to be undergoing apoptosis at a greater extent relative to the controls and the 50 mg/kg (LD) treated groups. This is noted by the number of stained dark brown nuclei seen in Figure 1. Also, Figure 1b (2 weeks LD) and 1f (4 weeks LD) showed more blue (nonapoptotic) nuclei than the other figures.

Quantification of apoptosis via measuring Casp3 activation

Liver apoptosis was evaluated by the Casp3 activity assay. The Casp3 activity in whole liver homogenates was measured by luminogenic Casp3 substrate optimized for Casp3 activity. The mean consequential luminescence is proportional to the amount of caspase activity generated [Figure 2a]. The HD animals in the 14- and 28-day exposure-periods showed a significantly greater hepatic Casp3 activity in comparison with the control rats. The LD and MD animals in the 2-week exposure group had a not significantly slightly higher caspase activity relative to the control group animals. Within the 4-week treated group, Casp3 activity was significantly greater for the MD and HD animals. There was no correlation noted between the hepatic caspase activity as measured by the luminescence assay and differentially expressed caspase mRNA gene expression fold analysis in the liver [Figure 2b].

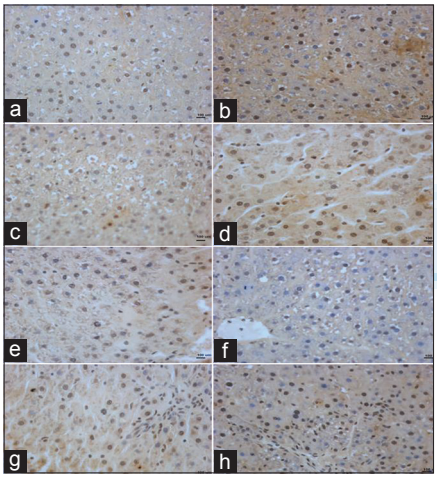


Figure 1: TUNEL apoptosis detection in the liver of F344 rats exposed to 2AA for 14- and 28-days. Apoptotic nuclei were stained dark brown. Animals were treated to 2AA contaminated diet for 14 days: (a) control – 0 mg/kg, (b) 50 mg/kg, (c) 75 mg/kg and (d) 100 mg/kg. Twenty eight days group included: (e) control – 0 mg/kg, (f) 50 mg/kg, (g) 75 mg/kg and (h) 100 mg/kg

Differential mRNA expression of selected apoptosis-related gene transcripts

The expressions of six mRNAs that play essential roles in modulating the apoptotic events in the cell were analyzed. With respect to the genes studied, there seem to be greater gene expression levels of treated groups when compared with control animals for both periods [Figure 3]. The HD (100 mg/kg-diet) and MD (75 mg/kg-diet) animals showed greater gene expression values for all transcripts. On the contrary, Casp3 was the least expressed in all treatment groups. The expression of Casp3 was less in the treatment groups than in the control animals. Aside from the AEN gene, the 2-week exposed rats had higher differentially expressed genes than the 4-week treated rats. BAX had greatest mRNA expression in the 2-weeks group. Similarly, AEN was the most expressed gene in the 28-day set. MDM2 and c-JUN were also highly expressed in the 2-week treated animals. For the 2-week class, AEN was at least two-fold changed in LD, MD, and HD samples for both treatment periods. BAX was at least two-fold expressed in LD, MD, and HD for the 4 week treatment period. It was only at least two-fold changed in the HD group for the 2-week class. JUN was at least 10-fold expressed in MD and HD, and at least two-fold changed in LD and MD for the 14- and 28-day exposure periods, respectively. MDM2 was also at least 12-fold changed in LD, MD, and HD during the 2 weeks of exposure to

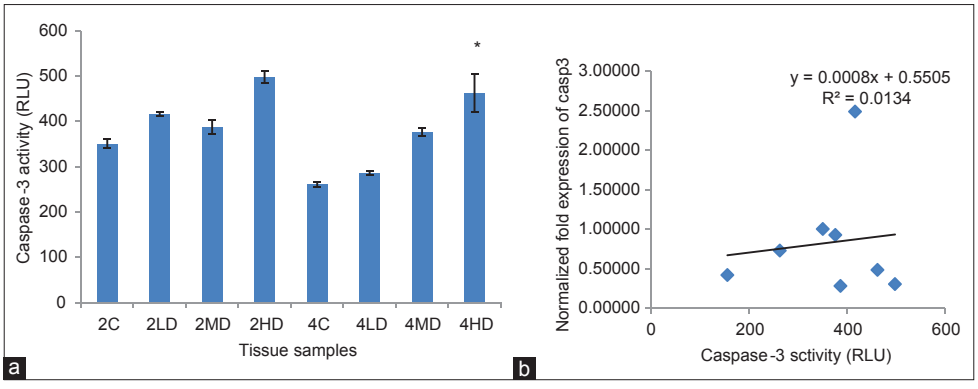


Figure 2: (a) Measurement of caspase-3 activity as luminescence in relative light units (RLU) mean ± STD. (b) Correlation plot of hepatic caspase activity verses quantified mRNA expression levels

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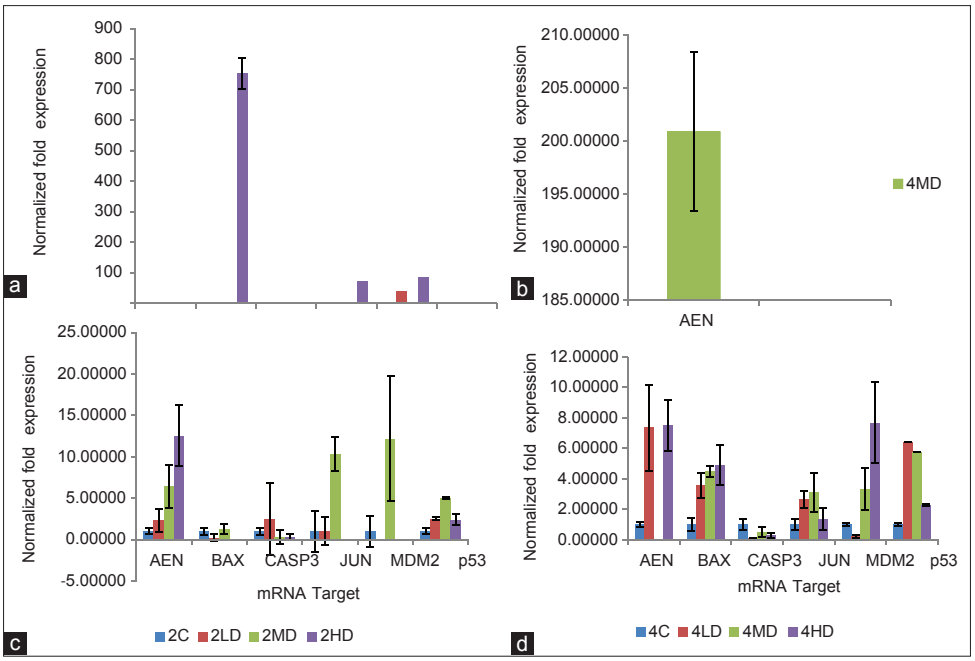


Figure 3: Hepatic expression of selected apoptotic genes in response to 2AA-contaminated dietary consumption. Normalized fold expression of mRNA transcripts from 14-day and 28-day treated rats represented in a and b respectively

2AA. With respect to the 28-day group, MDM2 was only three-fold expressed in the MD and HD animals. Similarly, p53 was at least two-fold altered in LD, MD, and HD rats for both time frames.

DISCUSSION

Apoptosis is the natural and essential process by which unwanted or damaged cells are cleared from tissues.[22] This is a genetically-controlled process that a cell undergoes in response to various stress conditions including ionizing, radiation, and exposure to various environmental contaminants such as aflatoxin.[23-25] The present study is an investigation of the role of apoptosis in minimizing the effects of dietary 2AA exposure. Apoptotic events are part of regulated cellular processes during development and aging intended to maintain cellular and organ tissue integrity. Apoptosis also occurs as a homeostatic and defense mechanism against various stress agents.[26]

The apoptotic events were examined through the detection of apoptotic cells via immunohistochemical TUNEL staining and Casp3 activity assay. Specific apoptosis staining seems to show greater level of apoptosis in MD and HD 2AA treated animals relative to their controls. This is noted because the dark brown staining of nuclei is more prominent in the livers of animals that consumed the 75 and 100 mg/kg-diet of 2AA. However, a further analysis using 'number of TUNEL index' was inconclusive. The reason for this anomaly might be the freeze-thaw artifacts of liver samples used in the study. Measured Casp3 activity indicated caspase activity in all hepatic tissues. Within the 14-day group, caspase activity was only significantly elevated in the HD animals. Similarly, increased caspase activity was noted in the medium- and HD group animals that were exposed to 2AA for 28 days. Coupled with these assays, was quantitative mRNA expression levels analyzed via qRT-PCR. The expressions of some hepatic apoptotic genes were determined to gain insight into how 2AA modulates AEN, BAX, CASP3, JUN, MDM2, and p53 to trigger apoptosis. Brief descriptions of these genes are provided below.

AEN, also called apoptosis-enhancing nuclease, is a target of the important p53 gene. AEN is reported to be a typical exonuclease and its expression is regulated by the phosphorylation of p53 transcript.[27] AEN has been reported to be highly expressed in animals exposed to various contaminants. For example, in rats exposed to genotoxic chemicals, there was increased incidence of apoptosis that correlated with AEN expression. A high level of AEN was also expressed in the livers of barrows fed dietary aflaxtoxin. Ionizing radiation was also reported to induce AEN expression.

We also investigated whether or not 2AA modulates the expression of Casp3 gene. Casp3 has been observed to be frequently activated in mammalian cell apoptosis.[28-30] This protein belongs to a family of cysteine proteases. Casp3 has been observed to be the most essential of the executioner caspases. It is activated by any of the initiator caspases such as caspase 8, 9, or 10.[26-31] Apoptosis has also been observed independent of caspase activation.[32]

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BAX gene is reported to induce apoptosis.[28,33-35] The BAX protein belongs to a family of Bcl2 intracellular proteins that regulate the activation of caspase.[36] These proteins have been found in many cancerous tissues.[37,38] The BAX gene is found in the cytosol of many cells in the inactive state. It responds to death stimuli by undergoing conformational change followed by transport of the BAX to the mitochondrial membrane. In the process, the BAX inserts and promotes release of apoptogenic genes.

JUN was the fourth gene whose expression was examined. This gene is a signal transduction transcription factor belonging to the AP-1 family.[39,40] The proto-oncogene has been observed to play modulating roles in cell proliferation and differentiation.[39,41] Studies have linked apoptosis with c-JUN expression. Recent studies have found high expression of proto-oncogene c-JUN in apoptotic tissues.[42-44]

We also analyzed the expression of murine double minute-2 in liver tissues. MDM2 works in tandem with p53 to regulate cell cycle and apoptosis. Direct protein-protein interaction between MDM2 and p53 leads to the regulation of p53. Besides MDM2's modulation of p53 tumor suppressor protein, MDM2 is also observed to exhibit anti-inflammatory effects. The anti-inflammatory effects are a result of MDM2 acting as co-transcription factor for nuclear factor-kappa-light-enhancer of activated B cells (NF-κB) at cytokine promoters.[45-48]

The last gene to be analyzed was p53, which is considered the guardian of the genome.[49] p53 is reported to play a significant role in preventing the development of cancers. This is accomplished via p53's ability to either potentially arrest or kill tumor cells.[50,51] As a result, it has been observed that at least half of all cancers do have loss of p53 activity from mutations in the p53 gene. The p53 protein also induces apoptosis and cell cycle arrest.[52]

The current results seem to show AEN and BAX as the main targets in the induction of apoptosis in response to 2AA exposure, though p53, MDM2, and JUN may play marginal roles. BAX was very highly expressed in the HD rats belonging to the 2-week exposure group. This trend was not observed in the 4-week animals. Instead, AEN was rather very highly expressed in the liver of the MD animals that were treated with 2AA for 28 days. The shift in protein target was rather surprising. It should also be noted that the rest of the mRNA transcripts were at least two-fold changed relative to the control genes and samples.

We have previously noted that the MD and HD animals respond similarly to 2AA toxicity.[2] For instance, we have previously observed that cumulative body weight gain in response to 2AA dietary consumption was similar for MD and HD animals (Gato and Means, 2011a). These groups of rats showed greater numbers of differentially expressed genes relative to the control and the LD for both periods. This was the case in the current investigation. The control and LD animals also seem to have similar responses to 2AA intoxication. Even though this was the case, the LD (50 mg/kg) animals still showed a higher expressed MDM2 gene than what had been previously observed in our studies.

Our observations indicate an intrinsic mode of apoptosis for both time frames. Our findings suggest that the cell is adopting two complementary strategies to maintain cellular integrity. Whereas, the short-term group focused on using BAX protein as the main target, the long-term treatment used AEN as the focal point gene. The acute exposure to 2AA for 14 days seems to cause the cell to be overwhelmed with 2AA toxication. As a result, we believe that BAX was very highly expressed in the short-term exposure group in order that apoptosis may be enhanced. This occurred via MDM2 binding to p53 gene that in turn, activated the BAX proapoptotic protein. The p53 not only activated the BAX and other Bcl2 family proapoptotic proteins, but also inhibited antiapoptotic family of proteins. Thus, the cell is committed to disintegration. This observation is consistent with the current results. At least a two-fold expression of MDM2, p53, and BAX has been noted in almost all 2AA treated animals.

On the contrary, the 4-week rats seem to have adapted to the effects of 2AA. This is the primary reason that the BAX expression is significantly reduced in the long-term group when compared with the 2-week group. Rather, AEN was highly expressed along with moderate expressions of MDM2, JUN, and p53 being, at least, two-fold changed. AEN is reported to have the ability to induce apoptosis by itself.[27] But this gene is also a direct target of the p53 gene. We think both of these approaches are vital for the induction of apoptosis due to 2AA cellular injury.

CONCLUSION

The present investigation examines the response of F344 rats to 2AA dietary consumption. Apoptosis is a well-documented cellular strategy to either eliminate or minimize the effects of environmental stressors. Specific apoptosis staining image does not seem to indicate significant difference between control and treated animals. This may be due to freeze-thaw artifacts observed in the liver samples. However, there appears to be a greater level of apoptosis in the MD and HD 2AA treated animals. Measured Casp3 activity indicated caspase activity in all hepatic tissues with significant activity in the HD group for the 14- and 28-day treatment groups. Analyses of apoptosis-related genes seem to show AEN and BAX as the main targets in the induction of apoptosis in response to 2AA exposure, though p53, MDM2, and JUN may play marginal roles. Dose-dependent

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increases in mRNA expression were observed in all genes except Casp3. BAX was very highly expressed in the HD rats belonging to the 2-week exposure group. This trend was not observed in the 4-week animals. Instead, AEN was rather very highly expressed in the liver of the MD animals that were treated with 2AA for 28 days.

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