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Joan Cheng April 11, 2017

Characterizing the Downregulation of CYP2J2 by Nitric Oxide

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Arachidonic acid (AA), antihistamine drugs, anticancer drugs, and other relevant drugs are metabolized by the cytochrome P450 enzyme CYP2J2. Its metabolism of AA is especially important, as the metabolites formed can protect against inflammation and hypertension but also promote carcinoma metastasis. Thus understanding how the enzyme is regulated within the cell, particularly by nitric oxide (NO), is pertinent.

In the human body, NO is produced in small amounts by three main isoforms of nitric oxide synthase. Overproduction has been linked to several disease states, and NO itself is a highly reactive molecule capable of permanently modifying macromolecules and inducing cell apoptosis. As a result, NO was thought to lack the specificity to participate in cell signaling events. However, evidence now points to the role of NO in signaling and regulation, particularly of P450 enzymes.

Experiments with Huh7-2J2v5, a human liver cancer cell line stably transduced with CYP2J2v5, reveal that NO donated from dipropylenetriamine NONOate (DPTA) downregulated CYP2J2 in a time and concentration-dependent manner. Since CYP2J2 mRNA levels were unaffected by NO, and the translational inhibitor cycloheximide was unable to block downregulation, it was concluded that protein degradation was occurring. Although preliminary data showed that the proteasomal inhibitor bortezomib was able to partially restore CYP2J2 levels, suggesting an ubiquitination-dependent pathway, high molecular weight (ubiquitinated) species were not observed. Furthermore, additional experiments concluded that bortezomib as well as the autophagy inhibitors 3-methylamine and chloroquine were not actually able to block downregulation. Thus downregulation occurs through neither an ubiquitin-dependent nor lysosomal pathway.

Once the mechanism is fully characterized by further studies using other protein degradation inhibitors, it will be tested to see whether it occurs in cardiac myocytes, where CYP2J2 is expressed a naturally higher levels. Overall, these findings are significant to understanding how to treat various disease states related to abnormal CYP2J2 and/or NO expression.

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Table of Contents

1. INTRODUCTION

1.1. Cytochrome P450 and CYP2J2

In order to pursue the development and improvement of pharmaceutical drugs, understanding the enzymes that metabolize these drugs is key. The most important ones to understand are the cytochrome P450s (CYPs), proteins that account for 75% of the total drug metabolism that occurs in the human body (Guengerich, 2008). As phase I enzymes, they are responsible for the oxidative biotransformation of most drugs and other lipophilic xenobiotics (Lewis, 2003). Thus knowing the mechanisms by which CYPs interact with and are regulated by other molecules is significant to clinical pharmacology.

CYPs are a superfamily of heme-containing monoxygenases that are denoted by the family (numerical designation), subfamily (letter designation), and individual gene (numerical designation) that they belong to (Guengerich, 1992; King, 2009) (**Fig. 1.1**). Although most CYPs are primarily expressed in hepatocytes, CYP2J2 is expressed at higher levels in the heart and endothelial cells than in the liver cells (Delozier et al., 2007, Michaud et al., 2010).

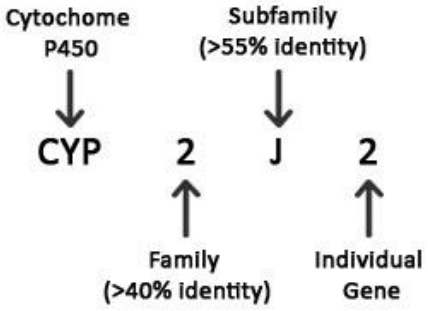

Figure 1.1. Nomenclature for cytochrome P450s, using CYP2J2 as an example.

This protein is the sole member of the human CYP2J subfamily and one of the major P450 enzymes to metabolize arachidonic acid (AA) into all four regioisomeric *cis*-epoxyeicosatrienoic acids (EETs) via NADPH-dependent olefin epoxidation (Zanger and Schwab, 2013). These metabolites subsequently play important roles in protecting against acute and chronic inflammation, as they exhibit a broad spectrum of anti-inflammatory activity such as suppressing the transmission of pro-inflammatory signals from cardiomyocytes to macrophages in the heart, preventing cardiac fibrosis, or reducing hepatic inflammation via the PPARγ which attenuates insulin resistance (Shahabi et al., 2014; Yang et al., 2015; Li et al., 2014). EETs

additionally protect against hypertension, as they are capable of producing vasorelaxation in a number of vascular beds by activating the smooth muscle large conductance Ca^{2+} activated K^+ (BK_{CA}) channels and hyperpolarizing the smooth muscle (Hu and Kim, 1997; Kroetz and Zeldin, 2002).

However, CYP2J2 and other CYP epoxygenases also play a role in promoting carcinoma metastasis. It has been reported that CYP2 epoxygenases are overexpressed in human cancer tissues and cancer cell lines, and that EETs enhance tumor growth, increase carcinoma cell proliferation, and prevent apoptosis of cancer cells (Jiang et al., 2005). Additionally, CYP epoxygenases overexpression or EETs treatment promotes tumor metastasis independently of their effects on tumor growth (Jiang et al., 2007).

Aside from AA, CYP2J2 also metabolizes the antihistamine drugs terfenadine, astemizole, and ebastine, the anticancer drug tamoxifen, the anthelmintic drug albendazole, and other relevant drugs such as thioridazine or cyclosporine (Evangelista et al., 2013; Zanger and Schwab, 2013; Wu et al., 2013). Due to their role in these various metabolic activities, understanding their regulation within the cell is highly pertinent.

1.2. Nitric Oxide as a Regulator

In the human body, NO is produced from L-arginine by the three main isoforms of nitric oxide synthase, or NOS (Pfeilschifter, 2003) (**Fig. 1.2**). These isoforms are epithelial NOS (eNOS), which is related to vasodilation and vascular regulation, neuronal NOS (nNOS), which is linked to intracellular signaling, and inducible NOS (iNOS), which has a variety of situational functions (Adam, 2015). eNOS and nNOS production of NO is tightly regulated by calcium via a calmodulin-dependent mechanism (Murad, 1996). Similarly, iNOS expression is tightly regulated by well-characterized signal pathways including mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinases / signal-transducer-and-activator-of-transcription proteins (JNK/STAT) (Jung et al., 2007).

Physiological concentrations are typically low, ranging from 100 pM to 5 nM (Hall and Garthwaite, 2009). However, enhanced production of NO—due to inflammatory stimuli increasing iNOS abundance—leads to the inhibition of SIRT1, a protein deacetylase that typically limits apoptosis and inflammation by deacetylating p53 and p65. This results in the subsequent induction of transcription factor p53 and nuclear factor κB, leading to induced apoptosis and increased expression of proinflammatory genes (Shinozaki et al., 2014). Thus the pathogenesis of several disorders such as type 2 diabetes or Parkinson's disease is observed.

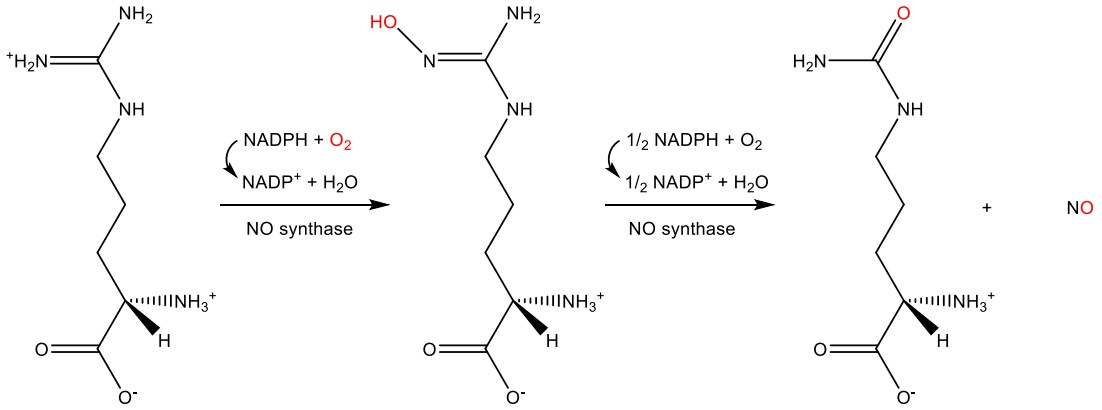

Figure 1.2. The reaction pathway for the production of NO from L-arginine.

Furthermore, NO is highly reactive and can permanently modify macromolecules, typically through oxidative damage (Pacher, 2007). In particular, peroxynitrite produced through the diffusion-limited reaction of NO and superoxide can induce cell apoptosis through irreversible protein nitration (Nauser, 2002; Sajad, 2013; Jope, 2000). It is for these reasons that NO was thought to lack the specificity to participate in cell signaling events. However, current evidence suggests that these post-translation modifications can not only be reversible but also play a role in redox signaling and signal transduction pathways.

When produced in a controlled manner, NO can covalently adduct to specific amino acids, like tyrosine or cysteine, to elicit a cellular effect (Wall et al., 2012) (**Fig. 1.3**). Tyrosine nitration was initially thought to be irreversible, but the discovery of a repair mechanism for nitrated proteins has pointed to the fact that tyrosine nitration can be reversed (Gow et al., 2004). In particular, tyrosine nitration plays an important role in activating or inhibiting regulatory proteins by preventing tyrosine phosphorylation (Lajtha and Gibson, 2007). As for

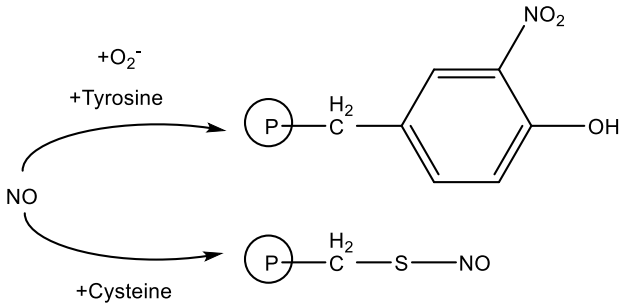

Figure 1.3. The reaction pathway for the nitrosylation of cysteine or nitration of tyrosine.

cysteine nitrosylation, it is more easily reversible and has been associated with the regulation of transcription factors, caspases, receptor tyrosine kinases, protein tyrosine phosphates, and other proteins (Yakovlev and Mikkelsen, 2010; Gould et al., 2013).

Even irreversible tyrosine nitration can play a role in long-term signaling for physiological processes such as myocyte differentiation during fetal heart development (Raoul, 2002). Furthermore, NO is implicit in the downregulation of many proteins. For example, although at low concentrations NO increases the activity of soluble guanylyl cyclase (sGC), at high concentrations it decreases sGC subunit mRNA and β_1 subunit protein levels via a cGMP-dependent mechanism, as well as reduces its β_1 subunit stability (Ferrero and Torres, 2002). Previous work in the lab has also shown that NO generated by iNOS and NO-donating chemicals is partially responsible for the downregulation of several P450 enzymes in hepatocytes in response to inflammatory stimuli. These enzymes include CYP2B1, CYP3A2, and CYP2B6, CYP2C22, and CYP2J2.

For CYP2B1, CYP3A2, and CYP2B6 in human cells, NO exposure results in their ubiquitination and subsequent rapid degradation via the proteasome, while for CYP2C22 in rat cells, degradation appears to occur via different mechanisms (Lee et al., 2008; Lee et al., 2014). However, the mechanism for CYP2J2 has yet to be fully identified. In order to further understand how NO reacts with cytochrome P450 proteins to target them for degradation, this study aims to characterize this downregulation of CYP2J2 by NO.

1.3. Proposal

Initial experiments show that NO donated by dipropylenetriamine NONOate (DPTA), which has a half-life of three hours at 37 ^{o}C, decreases CYP2J2 protein levels. This downregulation will be reaffirmed through time and concentration dependent treatments with DPTA. Afterwards, it will be determined whether downregulation occurs before or after translation by measuring CYP2J2 mRNA levels and by using a translational inhibitor in treatments. If downregulation occurs after translation, it must be due to protein degradation. The degradation pathway involved in the CYP2J2 proteolysis will be determined by using inhibitors. For example, if degradation is ubiquitin-dependent, high molecular weight species will form in the presence of proteasome inhibitors. Once this mechanism is characterized in hepatocytes, it will be tested to see if it occurs in cardiac myocytes, which have a naturally higher expression of CYP2J2. These results will not only further the understanding of the ways in which NO regulates P450 enzymes but also may point to a role in mediating the pathogenic effects of inflammation in cardiac tissue for NO.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

Chemicals used for treatments include DPTA (Cayman Chemical Company; Ann Arbor, MI), cycloheximide (CHX, Sigma Aldrich; St. Louis, MO), bortezomib (Bort; LC Laboratories; Darmstadt, Germany), 3-methyladenine (3MA; Acros Organic; Geel, Belgium), and chloroquine (CQ; Sigma Aldrich; St. Louis, MO).

2.2. Cell Culture

Huh7 cells—a well differentiated hepatocyte derived human cellular carcinoma cell line—were obtained from Arash Grakoui's lab, verified by the Emory Integrated Genomics Core, and stably transduced with CYP2J2 v5 lentivirus by Dr. Lee. The cells were seeded onto 12-well or 24-well plates (Corning Life Sciences; Corning, NY). They were grown in a humidified incubator at 37 ^{o}C with 5% CO_2, 1X Dulbecco's Modified Eagle Medium (DMEM; Corning Life Sciences; Corning, NY), 10% fetal bovine serum (FBS; Atlanta Biologicals; Nocross, GA), and a 1:1000 dilution of Penicillin-Streptomycin (Life Technologies; Waltham, MA). Once cells had reached around 90% to 100% confluency, they were treated for their respective experiments. Further details on each treatment are written in the results section.

2.3. Protein Extraction and Western Blot

After treatment, media were removed and the cells were washed with cold 1X PBS buffer. Twenty minutes after adding 120 µL of lysis buffer (25 mM TRIS-Cl, pH 7.5, 0.15 NaCl, 0.15% SDS, 10% NP40, 1 mM EDTA) to each well, cell lysates were collected and centrifuged at 15000 xg, 4 ^{o}C for 10 minutes in the Centrifuge 5424 (Eppendorf; Hamburg, Germany). The resulting supernatants were collected for a Western blot.

Protein concentrations were measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA) according to the manufacturer's protocol. The results were analyzed using the FLUOstar OMEGA microplate reader (BMG LabTech; Cary, NC) and OMEGA Data-Analysis software. Equal amounts of protein along with 2.5 uL of Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories; Hercules, CA) were loaded onto 8 – 16% Criterion™ TGX™ Precast Gels (Bio-Rad Laboratories; Hercules, CA). Electrophoresis ran at 190 V for 45 minutes.

The contents of the gels were transferred onto nitrocellulose membranes (Bio-Rad Laboratories; Hercules, CA) using the GENIE(R) Electrophoretic Transfer set up (Idea Scientific; Minneapolis, MN). The membranes were then blocked for 30 minutes with Odyssey Blocking Buffer (Li-COR Biosciences; Lincoln, NE) and probed overnight at 4 ^{o}C with v5 (1:10000 dilution), actin (1:5000 dilution), and GADPH (1:10000 dilution) primary antibodies (Sigma Aldrich; St. Louis, MO). Following a few brief washes, the membranes were probed for an hour at room temperature with IRDye 800CW (anti-rabbit, 1:10000 dilution) and IRDye 680LT (anti-mouse, 1:10000 dilution) secondary antibodies (Li-COR Biosciences; Lincoln, NE). In the case of the IP samples, the membrane was probed with anti-hemagglutinin (HA, 1:1000 dilution) primary antibody (Santa Cruz Biotechnology; Dallas, TX) and IRDye 680LT secondary antibodies (1:10000 dilution).

Imaging was done using the Odyssey^{FC} Imaging System (Li-COR Biosciences; Lincoln, NE) with the setting as follows: exposure to 700 nm light for 2 minutes and then exposure to 800 nm light for 10 minutes. ImageStudio software was then used to estimate the intensity of the bands.

2.4. Reverse Transcriptase and Real-Time qPCR

After treatment, media were removed and the cells were washed with cold 1X PBS buffer. RNA was extracted from the cells using a Direct-zol RNA Miniprep Kit (Zymo Research; Irvine, CA) according to the manufacturer's protocol. RNA concentrations were measured using the 260/280 nm spectrometric method with a FLUOstar OMEGA microplate reader and OMEGA Data-Analysis software.

cDNA was synthesized from equal amounts of RNA using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems; Foster City, CA) according to the manufacturer's protocol. The reverse transcription was done in a GeneAMP$^{(R)}$ PCR System 2400 ver. 2.11 (PerkinElmer; Waltham, MA) under the following program: 25 ^{o}C for 8 minutes, followed by 37 ^{o}C for 120 minutes, 85 ^{o}C for 5 minutes, and then held at 4 ^{o}C.

Real-time PCR was carried out using SYBR Green PCR Master MIX (Applied Biosystems; Foster City, CA) and forward/reverse primers for CYP2J2 and GADPH (Integrated DNA Technologies; Coralville, IA). Primer sequences were obtained from previously published papers (**Table 2.1**). The real-time PCR was done in a MasterCycler realplex^{4} qPCR system (Eppendorf; realplex; Hamburg, Germany) under the following program: 95 ^{o}C for 2 minutes, followed by 45 cycles of 95 ^{o}C for 10 seconds, 58 ^{o}C for 20 seconds, and 72 ^{o}C for 30 seconds, and then 95 ^{o}C for 15 seconds, 75 ^{o}C for 15 seconds, a melting curve step for 12 minutes, and 85 ^{o}C for 15

seconds. The results were analyzed using the realplex software to obtain the threshold cycle values (C_t).

Table 2.1. The primer sequences.

Gene	Primer Sequence	Reference
CYP2J2	*Sense*: GCGAAGGGGGAAAAAAAAAAAAAAAAACGG CTGGGAGCGAGGCGGG *Antisense*: CCCGCCTCGCTCCCAGCCGTTTTTTTTTTTTT TTTTTCCCCCTTCGC	(Lee and Murray, 2010)
GADPH	*Sense*: TGCCAAGTATGATGACATCAAGAAG *Antisense*: AGCCCAGGATGCCCTTTAGT	(Lee et al., 2008)

2.5. Transfection and Immunoprecipitation

Once cells had reached 60 – 80% confluency in a 12-well plate, they were transiently transfected with pCMV-HA-ub to allow for expression of ubiquitin with an HA tag (Kamitani et al, 1997). Transfection was done using jetPRIME$^{(R)}$ (Polyplus Transfection; New York, NY) according to the manufacturer's protocol. The following day, the cells were treated for their experiment. Further details on the treatment are written in the results section.

After treatment, media were removed and the cells were washed with cold 1X PBS buffer. Fifteen minutes after adding 200 µL of lysis buffer to each well, cell lysates were collected and centrifuged at 15000 xg, 4 ^{o}C for 10 minutes in the Centrifuge 5424 (Eppendorf; Hamburg, Germany). The resulting supernatants were then combined by taking 120 µL from each triplicate, and 300 µL of the combined supernatants were used for immunoprecipitation. This was done using Anti-V5 Agarose Affinity Gel (Sigma Aldrich; St. Louis, MO) according the manufacturer's protocol, except half-and-half of binding buffer (1X PBS, 0.1 % SDS, 1% NP40) and 1X PBS was used instead of 1X PBS. Samples before and after immunoprecipitation (IP) were both used for Western blot.

2.6. Data Analysis and Statistics

For PCR, the average CYP2J2 C_t value was subtracted from the average GADPH C_t value for each sample (dC_t). The average dC_t value of the control group was then subtracted from each dC_t value ($avgddC_t$). The $avgddC_t$ value was then multiplied by -1 and used as the power by which a base of 2 was raised to (nor $avgddC_t$). Next, the average and standard deviation of the $avgddC_t$ value for each treatment was calculated. These values were expressed as relative percentages of the control by dividing them by the average of the nor $avgddC_t$ signal for the control group and multiplying by 100%.

For Western blot, the normalized signal for each sample was calculated by adding the product of its actin signal and the average GADPH signal for all samples in the experiment and the product of its GADPH signal and the average actin signal for all samples in the experiment. The sample's CYP2J2 signal was then divided by its normalized signal to obtain its normalized CYP2J2 signal. This was done to control for differences in gel loading and transfer efficiency between cells.

The average and standard deviation of the normalized CYP2J2 signal for each treatment was then calculated. These values were expressed as relative percentages of the control by dividing them by the average of the normalized CYP2J2 signal for the control group and multiplying by 100%. Differences between treatments were calculated using a one-way ANOVA test followed by a t-test or Tukey multiple comparisons test as appropriate in GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA). Significance was set at $p<0.05$. The value of n signified the number of repeated experiments.

3. RESULTS AND DISCUSSION

3.1. Effect of DPTA on CYP2J2 Protein Expression

Cells were treated with varying concentrations of DPTA and incubated for varying amounts of time, as shown in **Figure 3.1**. Triplicates were done for each treatment.

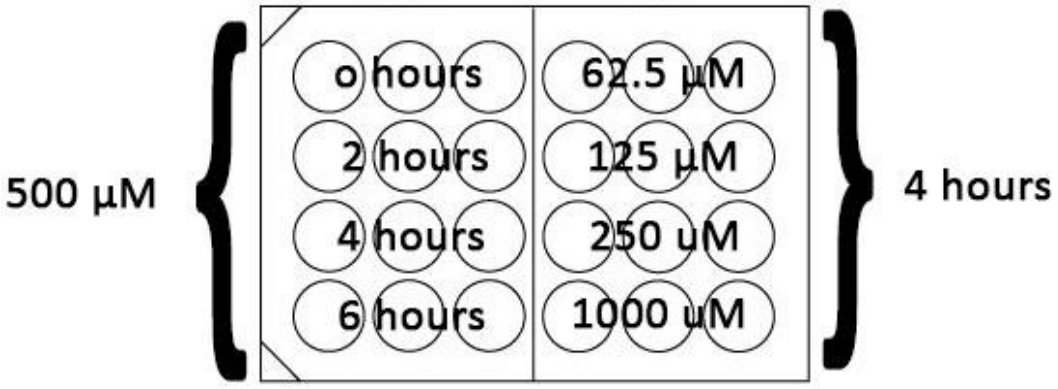

Figure 3.1. The time-concentration dependent treatment of Huh7 cells stably transduced with CYP2J2v5. Cells treated with different concentrations were incubated for 4 hours, while cells treated with 500 µM DPTA were incubated for different times.

Combined with data from identical treatments performed by Dr. Park, the results showed that CYP2J2 protein levels decreased in both a time-dependent and concentration-dependent manner, reaffirming preliminary findings that NO downregulates CYP2J2 expression (**Fig. 3.2a**). For the time-dependent treatment, it was concluded that the difference in protein levels was significant between 0 and 2 hours, 0 and 4 hours, 0 and 6 hours, and 2 and 4 hours (**Fig. 3.2b**). On the other hand, for the concentration-dependent treatment, the test concluded that protein levels differed significantly from the control but not from one another (**Fig. 3.2c**). Still, protein levels generally decreased with increasing concentration, except from 500 µM DPTA to 1000 µM DPTA.

One possible explanation for this unusual rise in protein levels is that at high concentrations, DPTA or one of its metabolites may bind to CYP2J2. Our hypothesis is that NO displaces a water ligand coordinated to CYP2J2's heme group, resulting in destabilization of the heme group and subsequent degradation of the enzyme (Li et al., 2004). Binding of DPTA or one of its metabolites to CYP2J2 may change its conformation, making the heme group inaccessible to NO. However, the error in the data point makes it difficult to assess if the results are significant or a statistical anomaly.

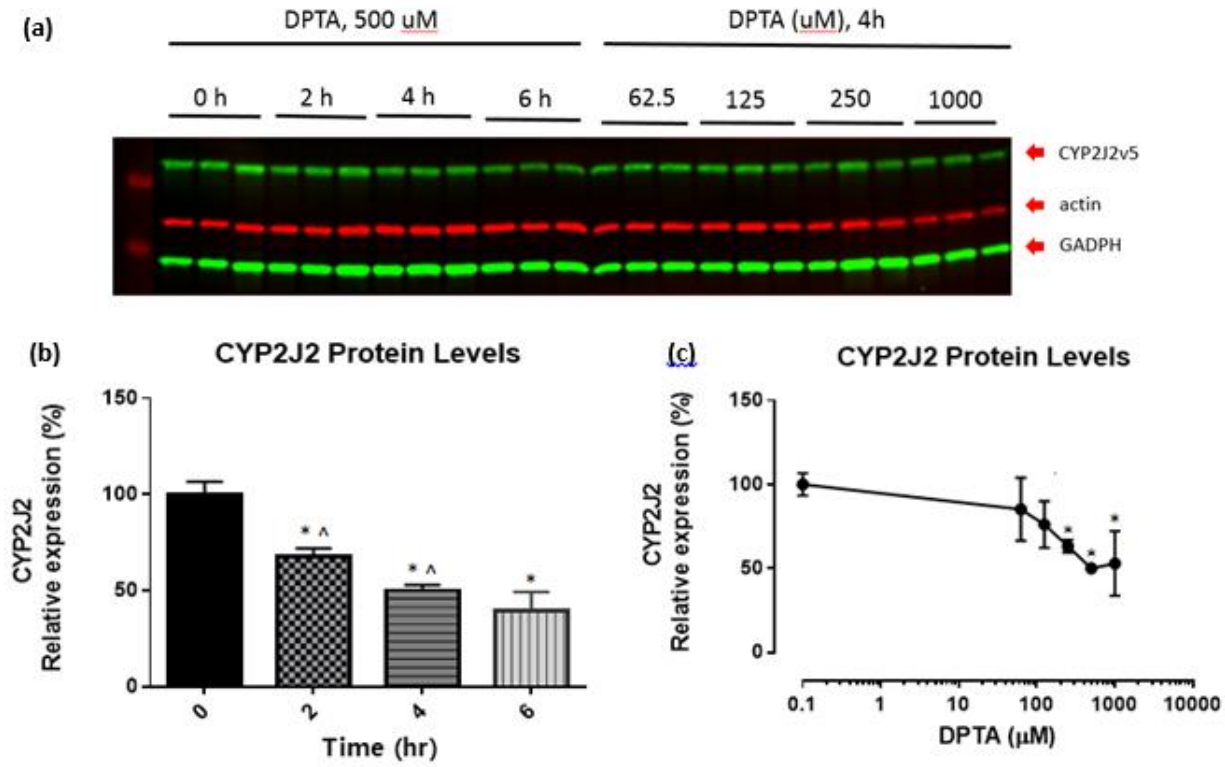

Figure 3.2. Effect of time and concentration on CYP2J2 protein expression (n = 3). Symbols [*] and [^] represent significant difference from the control and the column or data point to the left respectively ($p<0.05$). Triplicates were done for each treatment. Data was combined with Dr. Park's. (a) Western blot detection of CYP2J2, actin, and GADPH. (b) CYP2J2 protein levels for cells treated for different amounts of time, normalized to actin and GADPH protein levels. (c) CYP2J2 protein levels for cells treated with different concentrations of DPTA, normalized to actin and GADPH protein levels.

3.2. Effect of DPTA on CYP2J2 mRNA Expression

Cells were treated with 500 µM DPTA and incubated for 0, 2, 4, or 6 hours, as shown in **Figure 3.3**. Triplicates were done for each treatment.

No significant difference was found between any of the columns (**Fig. 3.4**). In fact, there was even an increase in mRNA levels for cells treated for 2 hours and 4 hours compared to cells treated for 0 hours. This suggested that downregulation is not transcriptional.

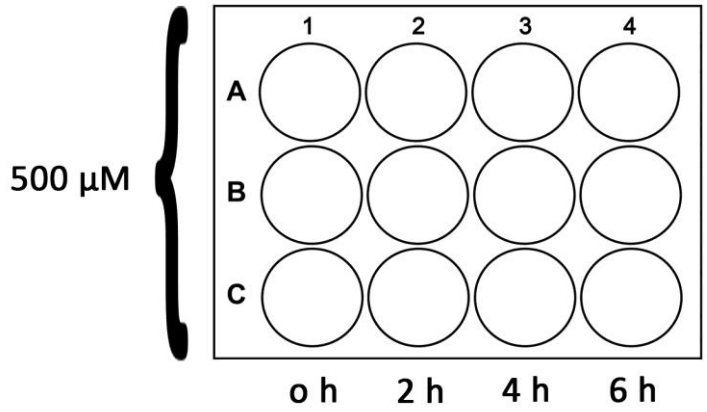

Figure 3.3. The time dependent treatment of Huh7 cells stably transduced with CYP2J2v5. Cells were treated with 500 µM DPTA and incubated for the times indicated.

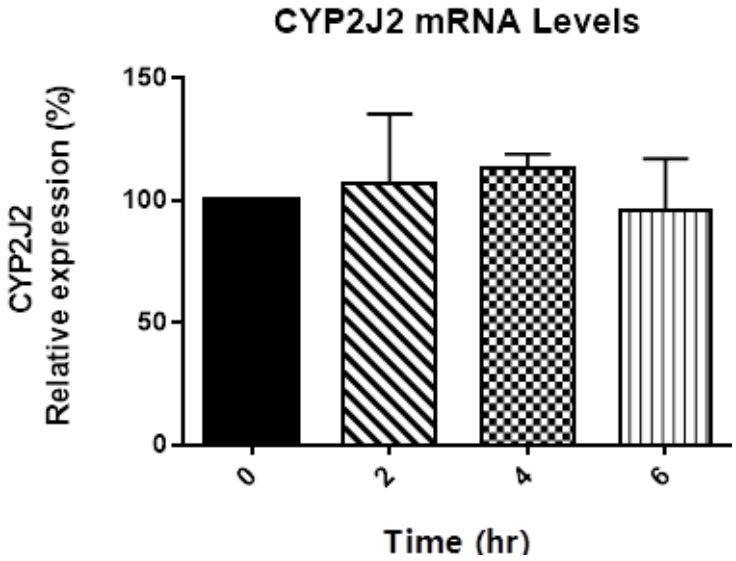

Figure 3.4. Effect of time on CYP2J2 mRNA expression for cells treated with 500 µM DPTA (n = 3). Triplicates were done for each treatment. No significant difference was observed between any of the columns ($p<0.05$).

3.3. Effect of DPTA on CYP2J2 Protein Expression for Huh7-2J2v5 Cells Treated With CHX

Cells were incubated with 10 µg/mL CHX for 30 minutes, after which half were treated with 500 µM DPTA. Cells were incubated for 0, 2, 4, or 6 hours, as shown in **Figure 3.5**, and triplicates were done for each treatment.

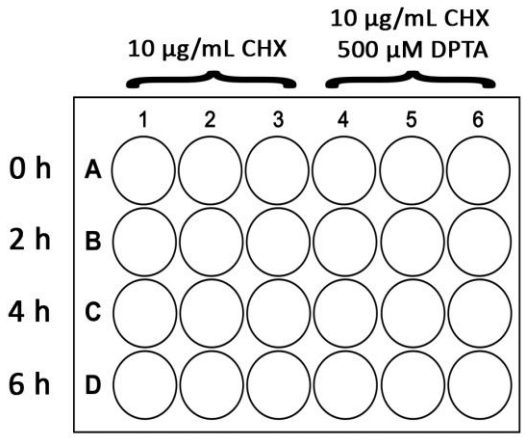

Figure 3.5. The time dependent treatment of Huh7 cells stably transduced with CYP2J2v5. Cells were treated with 10 µg/mL CHX with or without 500 µM DPTA and incubated for the times indicated.

Combined with data from identical treatments performed by Dr. Park, the results showed that CYP2J2 protein expression significantly decreased for cells treated with both CHX and DPTA compared to cells treated with only CHX (**Fig 3.6**). Furthermore, expression decreased in a time-dependent manner, reaffirming the results observed in **Figure 3.2b**. These observations, along with the observations in Section 3.2, pointed to downregulation occurring via protein degradation.

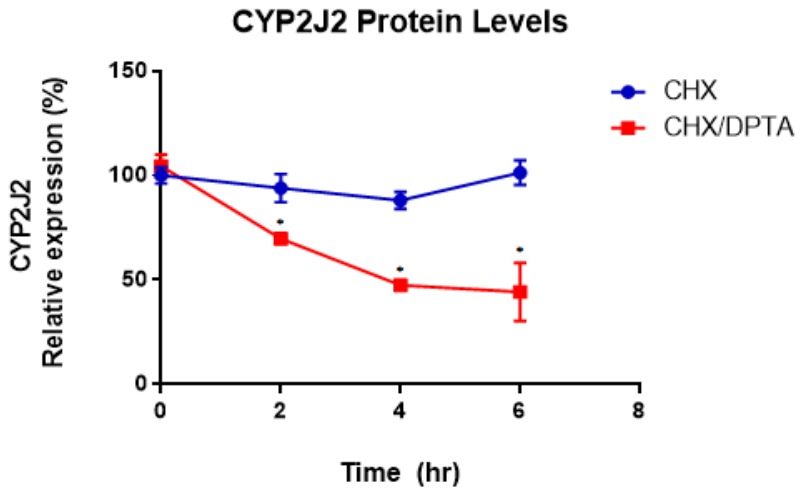

Figure 3.6. Effect of time on CYP2J2 protein expression for cells treated with 10 µg/mL CHX or 10 µg/mL CHX and 500 µM DPTA (n = 3). Triplicates were done for each treatment. Data was combined with Dr. Park's. The symbol [*] indicated that the protein levels for cells treated with CHX and DPTA were significantly different than the levels for cells treated with CHX for the same amount of time ($p<0.05$).

3.4. Detection of High Molecular Weight Species through Immunoprecipitation

The ubiquitin-dependent proteasome pathway is one of the ways in which protein can be degraded. In this pathway, the target protein is tagged through the covalent attachment of multiple ubiquitin molecules and subsequently degraded by the 26S proteasome, consisting of the catalytic 20S core and the 19S regulator (Lodish et al., 2004) (**Fig. 3.7**). Previous work done by Dr. Lee indicated that downregulation of CYP2B6 by NO followed such a pathway (Lee et al., submitted for publication). This can be seen in the appearance of a high-molecular weight species, i.e. HA-ub-CYP2B6, in the Western Blot for cells treated with DPTA (**Fig. 3.8**). As a proteasome inhibitor, bortezomib partially blocked the degradation of HA-ub-CYP2B6, resulting in their build-up. Thus a stronger signal was observed for cells treated with DPTA and bortezomib compared to cells treated with DPTA.

Preliminary results obtained by Dr. Park showed that bortezomib also partially restored CYP2J2 levels (**Fig 3.9**), implying that NO may cause CYP2J2 to be targeted for ubiquitination as well. Thus immunoprecipitation was performed in order to detect the presence of ubiquitinated, high molecular weight species.

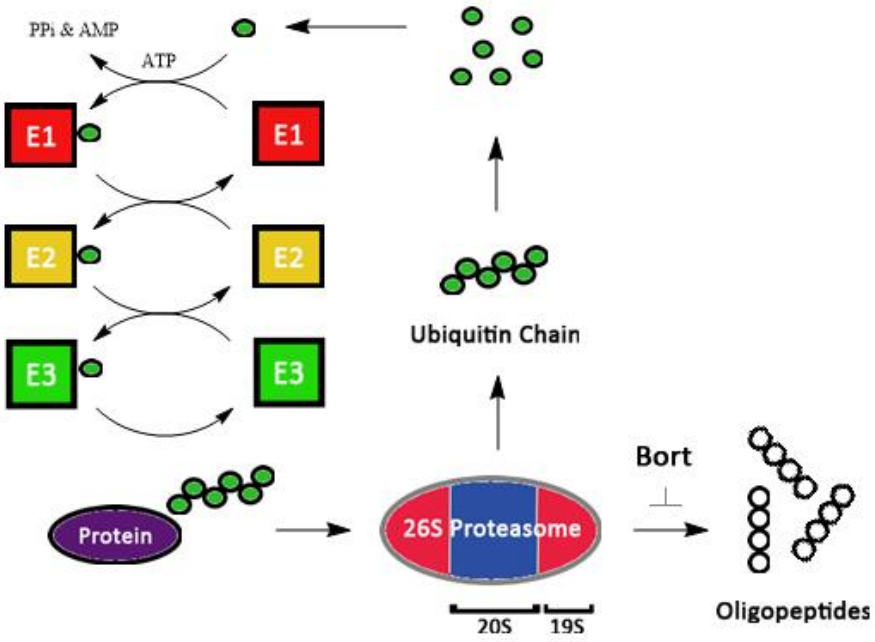

Figure 3.7. Diagram of the ubiquitin-dependent proteasome pathway.

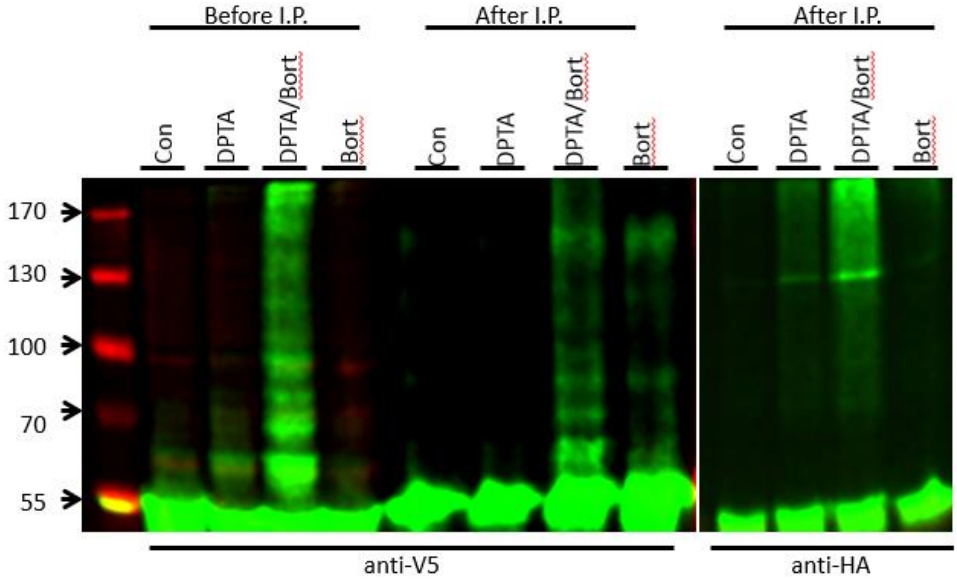

Figure 3.8. Western blot detection of CYP2B6, actin, GADPH, and HA-ub for Huh7 cells before and after immunoprecipitation with anti-V5 agarose gel. Smears from HA-ub-CYP2B6 were observed from 130 kD to 170 kD for cells treated with DPTA and cells treated with DPTA and Bort, with a stronger signal observed for the latter. Data was provided by Dr. Lee.

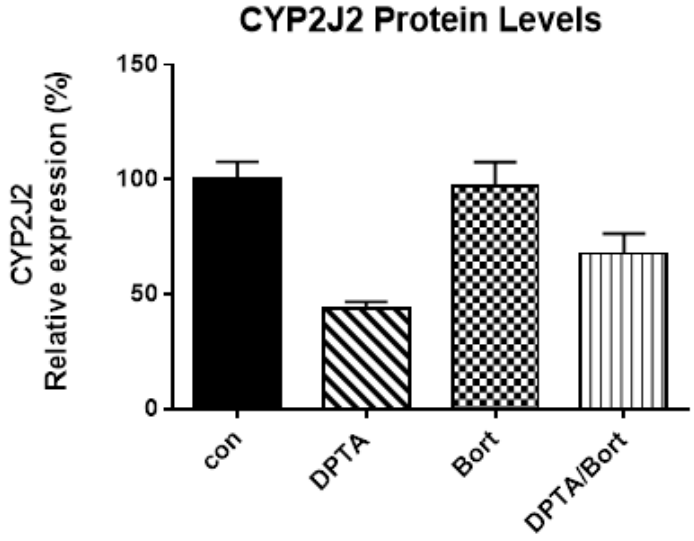

Figure 3.9. Effect of 10 µM Bort on CYP2J2 protein expression for cells treated with or without 500 µM DPTA (n = 3). Duplicates were done for each experiment. CYP2J2 protein expression noticeably increased for cells treated with both Bort and DPTA compared to cells treated with only DPTA. Data was provided by Dr. Park.

Cells transfected with pCMV-HA-ub were incubated with or without 10 µM bortezomib for 30 minutes before being treated with or without 500 µM DPTA for four hours as shown in **Figure 3.10**. Triplicates were done for each treatment.

After immunoprecipitation with anti-V5 agarose gel, the cells were observed for high molecular weight species through Western Blot as shown in **Figure 3.11**. However, the smear that was observed for HA-ub-CYP2B6 was not observed for HA-ub-CYP2J2. Furthermore, subsequent treatments with bortezomib concluded that the partial increase in CYP2J2 levels was not significant (discussed in Section 3.5). Thus protein degradation is most likely not ubiquitin dependent.

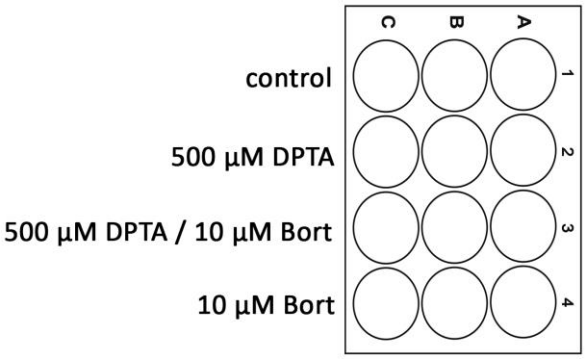

Figure 3.10. The Bort-dependent treatment of Huh7 cells stably transduced with CYP2J2v5 and transiently transfected with pCMV-HA-ub. Cells were incubated with or without Bort for 30 minutes before being treated with or without DPTA for four hours.

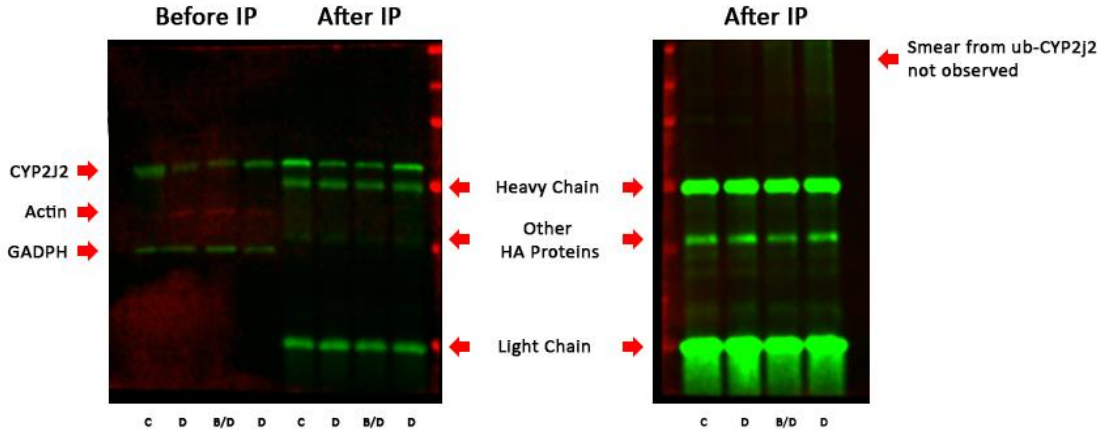

Figure 3.11. Western blot detection of CYP2J2, actin, GADPH, and HA-ub for Huh7 cells before and after immunoprecipitation with anti-V5 agarose gel. Bands for proteins modified with the surface glycoprotein HA were observed around 37 kD, but no smear from HA-ub-CYP2J2 was observed.

3.5. Effect of DPTA on CYP2J2 Protein Expression of Huh7-2J2v5 Cells Treated With Protein Degradation Inhibitors

Cells were incubated with or without the protein degradation inhibitor for their respective experiments for 30 minutes before being treated with or without 500 µM DPTA for four hours as shown in **Figure 3.12**. The concentration of the inhibitor depended on which one was being used (10 µM for bortezomib, 5 mM for 3MA, and 100 µM for CQ). Duplicates were done for each treatment, and treatments were done in 24-well plates such that three different experiments could be done per plate.

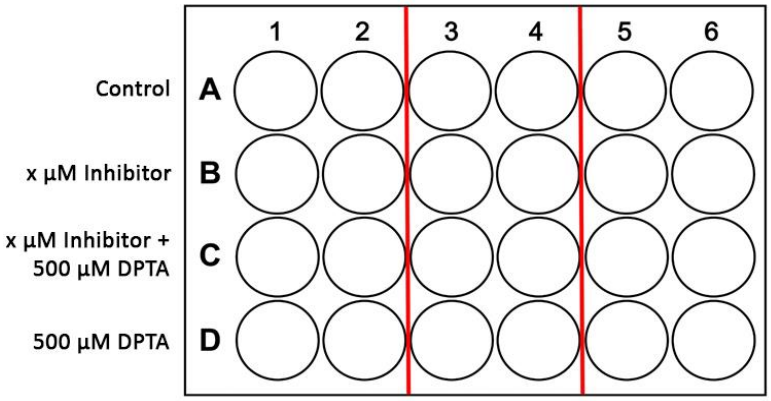

Figure 3.12. The inhibitor-dependent treatment of Huh7 cells stably transduced with CYP2J2v5. Cells were incubated with or without inhibitor for 30 minutes before being treated with or without DPTA for four hours. Inhibitor concentrations depended on the inhibitor. Experiments were separated as indicated by the red lines.

As stated before, bortezomib was used to test if protein degradation occurred via a proteasomal pathway (**Fig. 3.7**). While the same partial increase in CYP2J2 protein levels for cells treated with DPTA and bortezomib compared to cells treated with DPTA that had been observed in **Figure 3.9** was observed in **Figure 3.13**, the difference between the averages was revealed to not be significant. Combined with the results from Section 3.4, this points to degradation most likely not occurring through an ubiquitin-dependent proteasomal pathway.

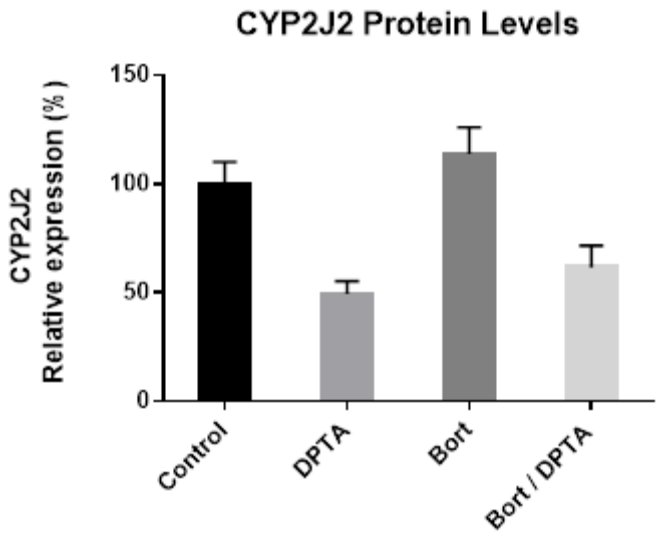

Figure 3.13. Effect of 10 µM Bort on CYP2J2 protein expression for cells treated with or without 500 µM DPTA (n = 5). Duplicates were done for each treatment. No significant difference was observed between cells treated with DPTA and cells treated with Bort and DPTA ($p<0.05$).

Another common pathway by which protein degradation occurs is the autophagy-lysosomal pathway, in which an autophagasome engulfs the target protein and subsequently fuses with a lysosome to degrade the protein in amino acids and fatty acids (Mizushina, 2007) (**Fig. 3.14**). 3MA is capable of blocking the autophagasome from engulfing the target protein whereas CQ is capable of blocking the autophagasome and lysosome from fusing. Thus these two inhibitors were used to test if protein degradation occurred via a lysosomal pathway.

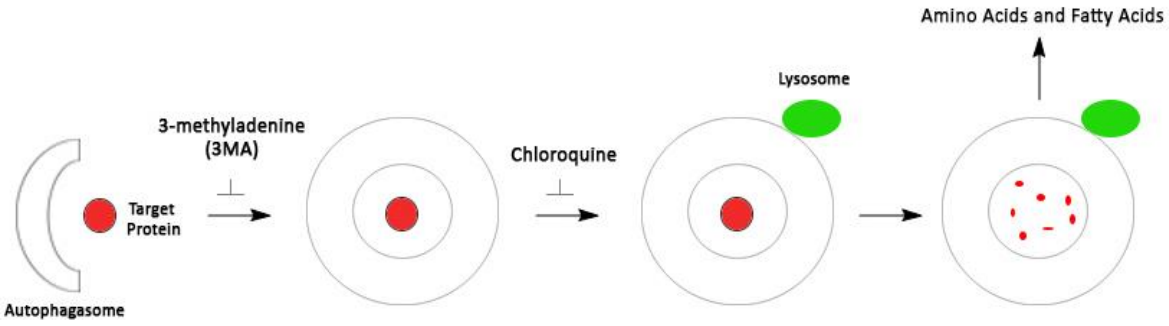

Figure 3.14. Diagram of the autophagy-lysosomal pathway.

CYP2J2 protein levels were partially restored for cells treated with 3MA and DPTA compared to cells treated with DPTA, but not to a significant degree (**Fig. 3.15**). Additionally, this partial restoration may have been observed for the same reason it was observed in cells treated with 1000 µM DPTA in Section 3.1. Cells treated with 3MA expressed less CYP2J2 than the control, and a Luciferin-2J2/4F12 activity assay—an assay which detects the rate at which CYP2J2 converts the bioluminescent probe into a luciferin ester—performed by Dr. Park showed that 3MA partially inhibited CYP2J2 activity in the cells (Ma et al., 2008) (**Fig. 3.16**). This points to 3MA potentially binding to CYP2J2, affecting its expression and function.

As for the other inhibitor, no difference was observed between cells treated with CQ and DPTA and to cells treated with DPTA (**Fig. 3.17**). Similar results were observed by Dr. Park when she repeated the experiments. Overall, this indicates that degradation is most likely not occurring through an autophagy-lysosomal pathway either.

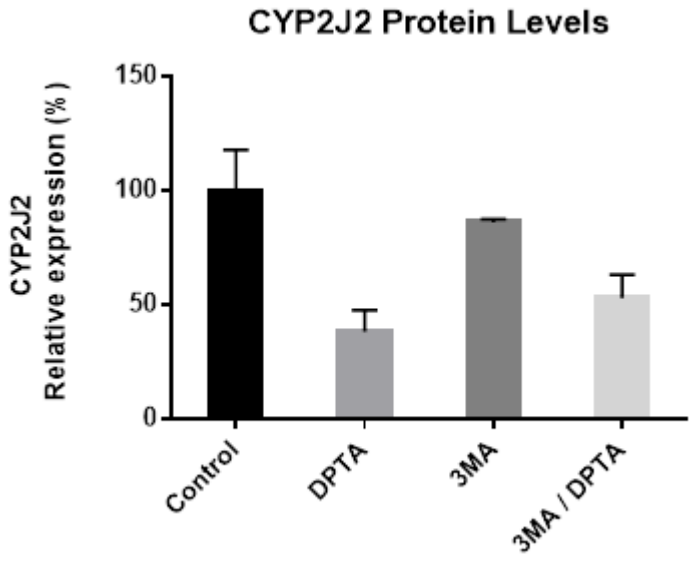

Figure 3.15. Effect of 5 mM 3MA on CYP2J2 protein expression for cells treated with or without 500 µM DPTA (n = 3). Duplicates were done for each treatment. No significant difference was observed between cells treated with DPTA and cells treated with 3MA and DPTA ($p<0.05$). A decrease was noted in protein levels for cells treated with 3MA compared to the control.

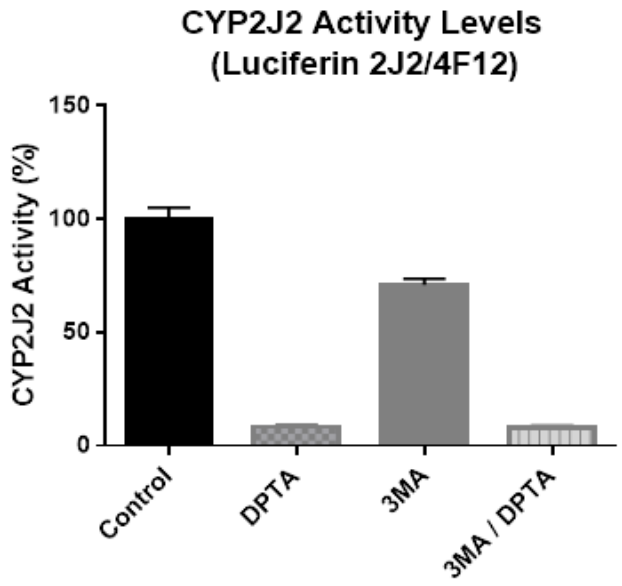

Figure 3.16. Effect of 5 mM 3MA on CYP2J2 activity for cells treated with or without 500 µM DPTA (n = 1). A decrease was noted in CYP2J2 for cells treated with 3MA compared to the control. Data was provided by Dr. Park.

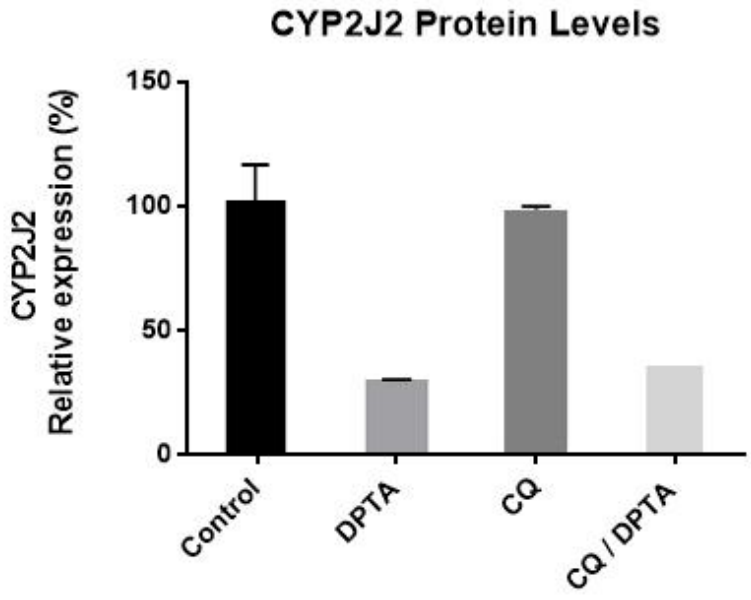

Figure 3.17. Effect of 100 mM CQ on CYP2J2 protein expression for cells treated with or without 500 µM DPTA (n = 1). No significant difference was observed between cells treated with DPTA and cells treated with CQ and DPTA ($p<0.05$).

4. CONCLUSION

The primary findings of this paper are as follows: (1) NO downregulates CYP2J2 protein expression in a time- and concentration-dependent manner, (2) this downregulation occurs via protein degradation, and (3) this degradation does not occur via an ubiquitin-dependent proteasomal pathway or an autophagy-lysosomal pathway.

From Section 3.1 and 3.2, it is observed that NO exhibits a time- and concentration-dependent downregulation of CYP2J2. Although CYP2J2 protein expression unexpectedly jumped up for cells treated with 1000 μM DPTA, the error in the data point makes it difficult to assess the results. If repeats of the concentration-dependent treatment confirm that an increase is truly occurring, then it may be worthwhile to determine if NO is coordinating to the heme group through Raman spectroscopy (Li et al., 2004) as well as test whether binding is causing a conformational change in CYP2J2 by performing treatments with other slow-releasing NO donors such as NOR-5 that have vastly different structures than DPTA.

While NO suppresses CYP1A1 expression at the transcriptional level (Stadler et al., 1994), the lack of change in CYP2J2 mRNA levels shows that this does not occur for CYP2J2. Furthermore, the decrease in CYP2J2 protein levels in spite of treatment with CHX also eliminate the possibility of translational regulation. Consequentially, the downregulation must occur through protein degradation.

For the CYP2B proteins, specifically 2B1, 2B2, and 2B6, NO exposure causes them to be targeted for ubiquitination and subsequent proteasomal degradation (Lee et al., 2007; Lee et al., submitted for publication). Yet the observed lack of high molecular weight species containing HA-ubiquitin in the blots from Section 3.4 as well as the inability of bortezomib to block downregulation by NO implies that ubiquitination does not occur. Similarly, 3MA and CQ were unable to block downregulation by NO, indicating that autophagy-lysosomal degradation does not occur either. In light of this, it seems CYP2J2 downregulation by NO occurs through a novel mechanism, much like CYP2C22 downregulation by NO (Lee et al., 2014).

Beyond downregulating CYP2J2, NO may also possibly inhibit CYP2J2 activity as it does with CYP1A1 and CYP2B1/2 (Wink et al., 1993). If so, NO may be able to suppress CYP2J2 far more potently than the current high-affinity, selective CYP2J2 inhibitors that exist (Lafite, Dijols, Zeldin, Dansette, & Mansuy, 2007). This can be tested with a Luciferin-2J2/4F12 assay.

These findings are crucial to understanding various disease states linked to abnormal CYP2J2 expression in the cell. Take for example chronic inflammatory bowel disease, where iNOS has been reported to be induced while CYP2J2 has been reported to be dysregulated (Singer et al. 1996; Bystrom et al., 2013). If iNOS induction can be proven to be the cause of

CYP2J2 dysregulation, then the findings of this paper can be used to formulate a way to circumvent CYP2J2 degradation by NO and allow the cells to combat the inflammation. Once the mechanism is fully characterized, it will be tested to see whether it occurs in cardiomyocytes, where CYP2J2 is naturally expressed at high levels.

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