EFFECT OF OMEGA-6 AND OMEGA-3 OXYLIPINS ON HNF4A PROTEIN STABILITY AND FUNCTION

By

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Abstract

Hepatocyte nuclear factor 4α (HNF4α) is a highly conserved nuclear receptor and has been implicated in many human diseases such as diabetes, hemophilia, and atherosclerosis. Previous unpublished research suggests that soybean oil, a popular cooking oil, may lead to decreased levels of HNF4α in the nuclei of hepatocytes. Soybean oil is rich in unsaturated fats, especially linoleic acid (C18:2), and assumed to be healthy, yet it induces obesity, diabetes, insulin resistance, and fatty liver in mice. Metabolomic analysis of the liver showed positive correlation between obesity and hepatic C18 oxylipin metabolites of omega-3 and omega-6 fatty acids (α-linoleic and linoleic acid, respectively) in the cytochrome P450/soluble epoxide hydrolase pathway. Studies also indicate that linoleic acid is an endogenous ligand for HNF4α, and computer modeling has suggested that it may be possible for oxylipins to bind HNF4α. Here, experiments utilizing cell culture and immunoblot analysis examined whether the presence of omega-3 and omega-6 oxylipins in cell culture destabilize HNF4α protein. The results suggest that the presence of oxylipins in media may destabilize HNF4α.

ACKNOWLEDGEMENTS

I would like to thank my faculty mentor, Dr. Sladek, for her exceptional guidance and mentorship throughout this project. I would also like to thank the other members of the Sladek lab, in particular Sarah Radi, for the teaching and assistance they provided me while in lab. It has been an honor to work alongside them. Additionally, I would like to thank the support of the MARC U STAR program. It is with the support of each of these individuals that I was able to complete a rewarding research experience at UCR and I am eternally grateful to them.

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INTRODUCTION

Hepatocyte Nuclear Factor 4α (HNF4α) is a highly conserved nuclear receptor, a type of transcription factor, which has been found in every animal examined thus far, from sponges to mice and humans. There are multiple isoforms of HNF4α in humans which originate from two promoters, P1 and P2. In humans, HNF4α regulates the expression of hundreds of genes, particularly those involved in metabolism, such as cytochromes P450 (*CYP*), and has been connected to multiple human diseases either directly through mutations in the *HNF4A* gene or indirectly through mutations in binding sites of the genes it regulates. The endogenous ligand of HNF4α was previously reported to be linoleic acid (LA), an essential fatty acid found in many food sources from sunflower seeds to soybean oil (Yuan et. al. 2009).

Soybean oil consumption in the United States has increased tremendously in recent years, becoming prominent in the Western diet due to its cheap cost and widespread use in commercial products and restaurants (Figure 1). Soybean oil has high amounts of LA, and as a result, consumption of this essential fatty acid has also increased tremendously. In the liver, LA is metabolized by CYP proteins, creating an epoxide. This epoxide is then hydrolyzed by soluble epoxide hydrolase *(EPHX2),* generating omega-3 and omega-6 oxylipins of LA (Figure 2).

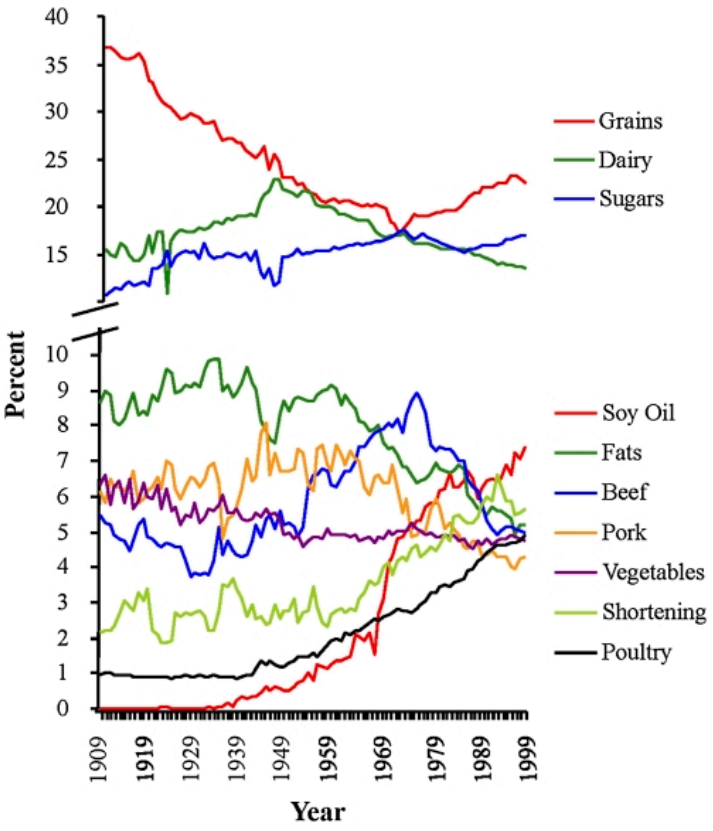


Figure 1 Percent consumption of various food products in the American diet between 1909 and 1999. (Blasbalg et. al. 2011)

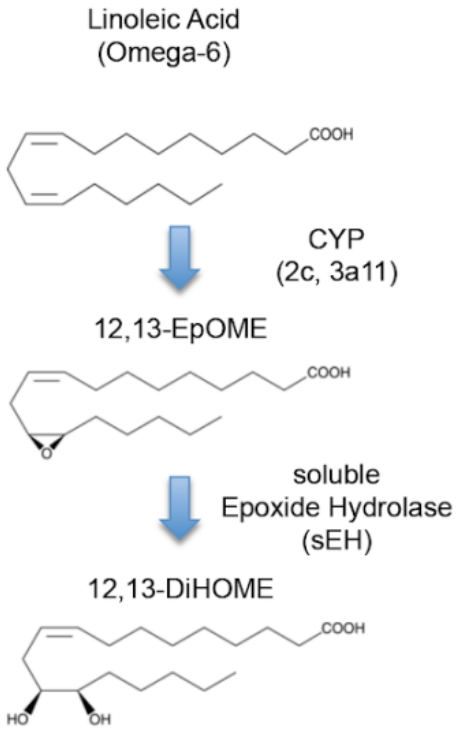


Figure 2 Conversion of linoleic acid to a 12,13-DiHOME oxylipin in the liver via CYP and EHX2 enzymes.

A previous study from the Sladek lab (Deol et. al. 2017) fed mice one of three diets in which 40% of kilocalories came from fats. The high fat diets were: CO (36% coconut oil, 4% soybean oil for essential fatty acids LA and α-linoleic), SO+CO (21% fat calories from coconut oil and 19 % from soybean oil), and PL+CO (conventional soybean oil was replaced with the genetically modified High Oleic Soybean Oil Plenish which is low in LA). In addition, mice were fed a control diet which had LA as 1.2% of the kilocalories in the diet. After 24 weeks on the diet, metabolic analysis of the liver found that oxylipins were positively related to body weight of the mice, with the most obese mice having the highest levels of oxylipins in the liver (Deol et. al. 2017). Oxylipins are a downstream metabolite of LA. Computer modeling of oxylipins and the ligand binding domain of HNF4α suggest that oxylipins may be able to bind in the ligand binding domain (not shown). Unpublished data show that the relative levels of HNF4α in the nuclei of mouse hepatocytes were reduced in mice that had higher levels of oxylipins in the liver. The lower levels of HNF4α in the hepatocyte nuclei and the possibility of oxylipins binding the ligand binding domain of HNF4α suggest that oxylipins may bind to and destabilize HNF4α. The goal of this project is to determine whether omega-6 and omega-3 oxylipins destabilized HNF4α using a cell-based assay.

METHODS

Cell Culture The human colorectal cell cancer cell line HCT116 (American Type Culture Collection [ATCC], CCL-247) was maintained in McCoy's 5A medium (Iwakata and Grace modification, with L-glutamine) (Corning Cellgro catalog no. 10-050-CV) supplemented with 10% fetal bovine serum (FBS) (BenchMark catalog no. 100-106) and 100 U/ml penicillin-

streptomycin (1% P/S). Cells were passaged at 85 to 95% confluence. Cells were genetically modified to express HNF4α2 upon addition of doxycycline using a Tet-On system (Vuong et. al. 2015): 1.5 X 10^6 cells were placed into each well of 6-well plates with McCoy's media. Once the cells were approximately 70% confluent, they were cultured in McCoy's media without serum and with 0.3 µg/mL doxycycline to induce expression of HNF4α2 through tetracycline-controlled transcriptional activation. After 24 hours, the cells were exposed to doxycycline (0.3 µg/mL), cycloheximide (50 µg/mL), and either dimethyl sulfoxide (DMSO) at a final concentration of 0.1% of media or oxylipins dissolved in DMSO with a final concentration of 10 µM of 9,10 DiHOME and 12,13 DiHOME oxylipins in media (a generous donation from Dr. Hammock at University of California- Davis). Cell extracts were collected using a RIPA lysis buffer (1 µM of DTT and 1 mM PMSF) at four time points (0 hours, 3 hours, 9 hours, and 12 hours) after exposure to either oxylipins or DMSO, then analyzed using immunoblotting. For each time point and condition, triplicate samples were analyzed.

Immunoblotting. For immunoblot (IB) analyses, protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred overnight to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore) at 22V using a wet transfer system. Signals were detected using the SuperSignal West Dura extended-duration substrate kit (Thermo Fisher Scientific); blots were exposed for 90 seconds using the Bio-Rad ChemiDoc system. A Bradford assay (Bio-Rad) was used to measure the protein concentration: 15 µg of whole-cell lysates were loaded into each lane. Blots were incubated for 30 minutes at room temperature in a blocking buffer (5% nonfat dried milk in 1X Tris buffered saline with Tween 20 (TBST)), then incubated in the same buffer but with primary antibodies at a 1:5,000 dilution at 15ºC for approximately 18 hours. Blots were then washed with TBST three times at

room temperature for 5 minutes each, then incubated with a secondary antibody at a 1:5,000 dilution for one hour at room temperature. Coomassie staining and immunoblot images for beta-actin of the blot were used to normalize protein loading. The primary antibodies were mouse monoclonal anti-HNF4 P1/P2 (R&D Systems, catalog no. PP-H1415-00) which recognize the C terminus of both the P1- and P2- HNF4α isoforms; and rabbit monoclonal β-actin (Cell Signaling, catalog no. 5125S). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies from Jackson ImmunoResearch Laboratories. Relative band intensities were measured using the volume tool of the ChemiDoc system.

Statistical analysis. Band intensities measured using the ChemiDoc system were normalized to protein on the blot either by normalizing to the Coomassie stain or beta-actin band. These values were then normalized to the average T=0 hour value of their respective immunoblot and a 2-way ANOVA test was utilized to compare these values between the two conditions over the course of 12 hours; multiple comparisons from the 2-way ANOVA was used to compare relative levels of HNF4α at each time point. Statistical significance is defined as a p value <0.05.

RESULTS

In order to determine whether oxylipins have an effect on the protein stability of HNF4α, cell-based assays using HCT116 cells engineered to express HNF4α upon addition of doxycycline were treated with cycloheximide, a protein synthesis inhibitor, and either DMSO, as a vehicle control, or a mixture of 9,10 DiHOME and 12,13 DiHOME oxylipins in DMSO. Protein samples were collected from multiple time points to examine possible changes in

decreasing levels of HNF4α over time between both conditions, as seen in the experiment design (Figure 3). The results from these experiments show that HNF4α may be destabilized by the presence of oxylipins in media (Figures 4-7).

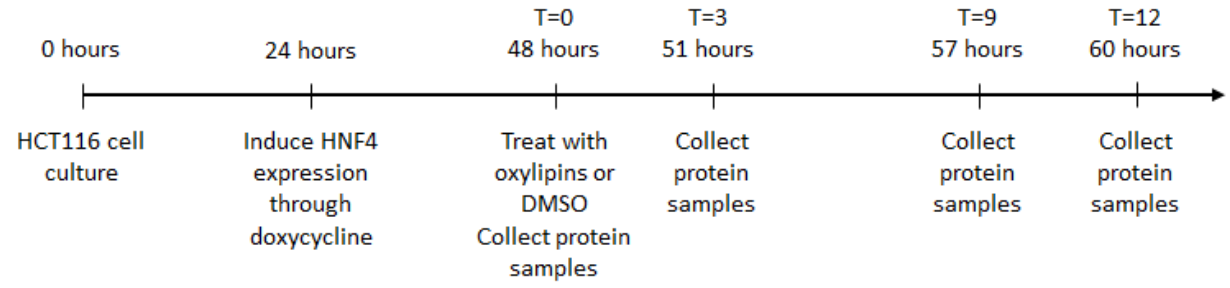


Figure 3. Experimental design

Genetically modified HCT116 cells were cultured in separate wells until they reached approximately 70% confluence. Human HNF4α2 expression was subsequently induced by the addition of doxycycline. After 24 hours, media was replaced and cells were exposed to doxycycline, cycloheximide, and oxylipins or DMSO. Protein samples collected at various time points after exposing cells to DMSO or oxylipins.

Immunoblotting can be utilized to answer whether a treatment destabilizes protein. To examine protein stability, cells were exposed to cycloheximide in addition to being exposed to doxycycline and either DMSO or oxylipins (Figure 3). Cycloheximide is a eukaryotic protein synthesis inhibitor and its presence in media prevents translation of additional HNF4α over the course of protein collection. As a result, relative levels of HNF4α protein levels should decrease over time in both treatments. The rate in which HNF4α protein levels decrease is compared between cells which are exposed to either DMSO or oxylipins to determine if cells exposed to oxylipins resulted in lower levels of HNF4α protein over the course of 12 hours (Figures 4, 5, 6 and 7).

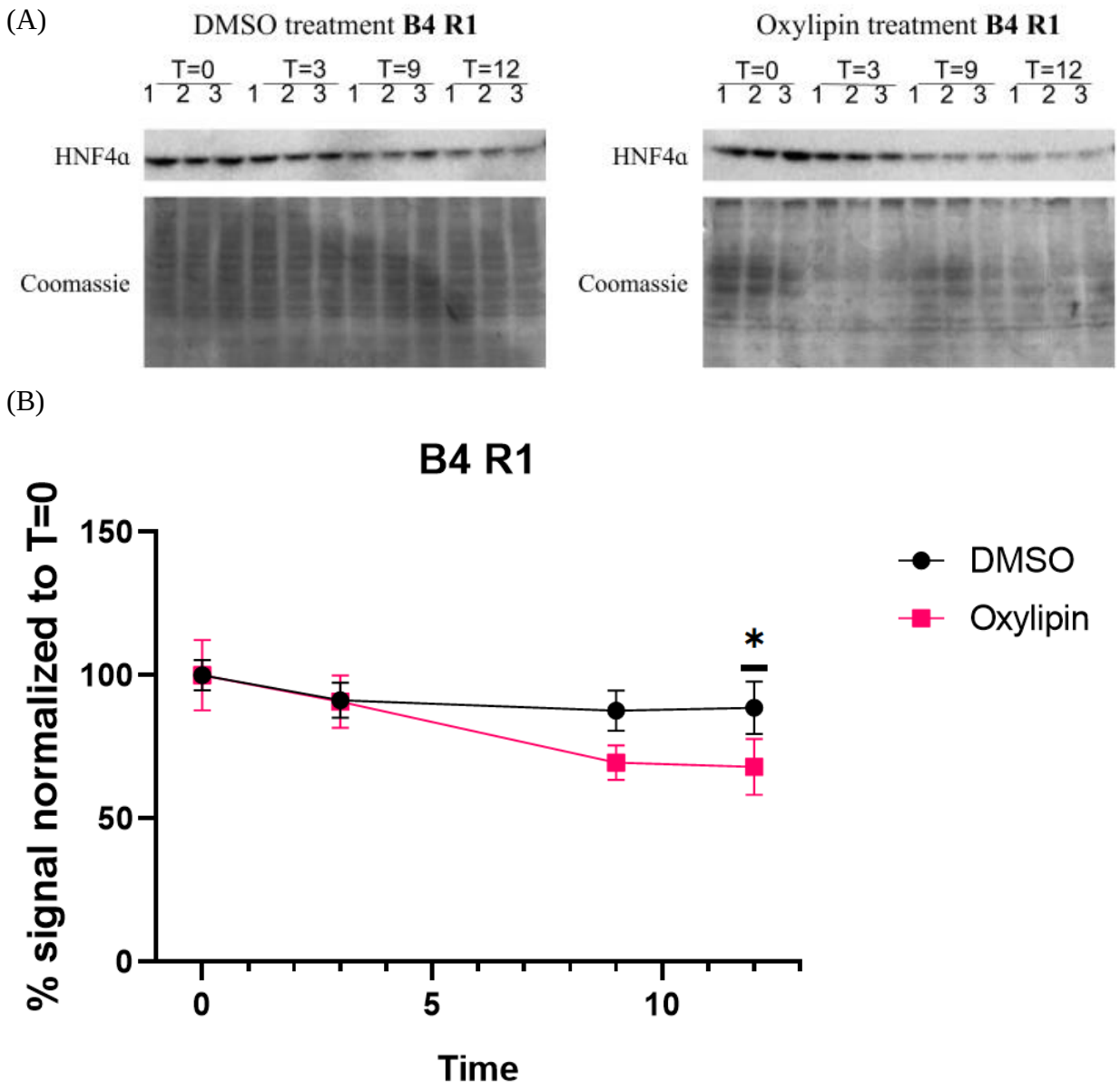


Figure 4 The presence of oxylipins in media may destabilize HNF4α.

(A) Immunoblot analysis of HNF4α P1/P2 in HCT116 cells induced by doxycycline and treated with DMSO (0.1%) or oxylipins (10 μM). (B) Graph of HNF4α signal normalized to Coomassie, then normalized to the average normalized value of the samples collected at 0 hours of exposure to DMSO or oxylipins. Multiple comparisons after 2-way ANOVA showed a significant difference in the two treatments at 12 hours of exposure (*p=0.0332). The immunoblots shown used samples from experiment number 4 (biological replicate 4, B4); this is the first analysis of those samples, technical replicate 1 (technical replicate 1, R1).

The presence of oxylipins in media may destabilize HNF4α The immunoblots in figure 4 show that over 12 hours, the relative levels of HNF4α protein in cells exposed to oxylipins are lower than the levels of HNF4α protein in cells exposed to DMSO, the vehicle control. Multiple comparisons after 2-way ANOVA showed a significant difference in the two treatments at 12 hours of exposure (*p=0.0332).

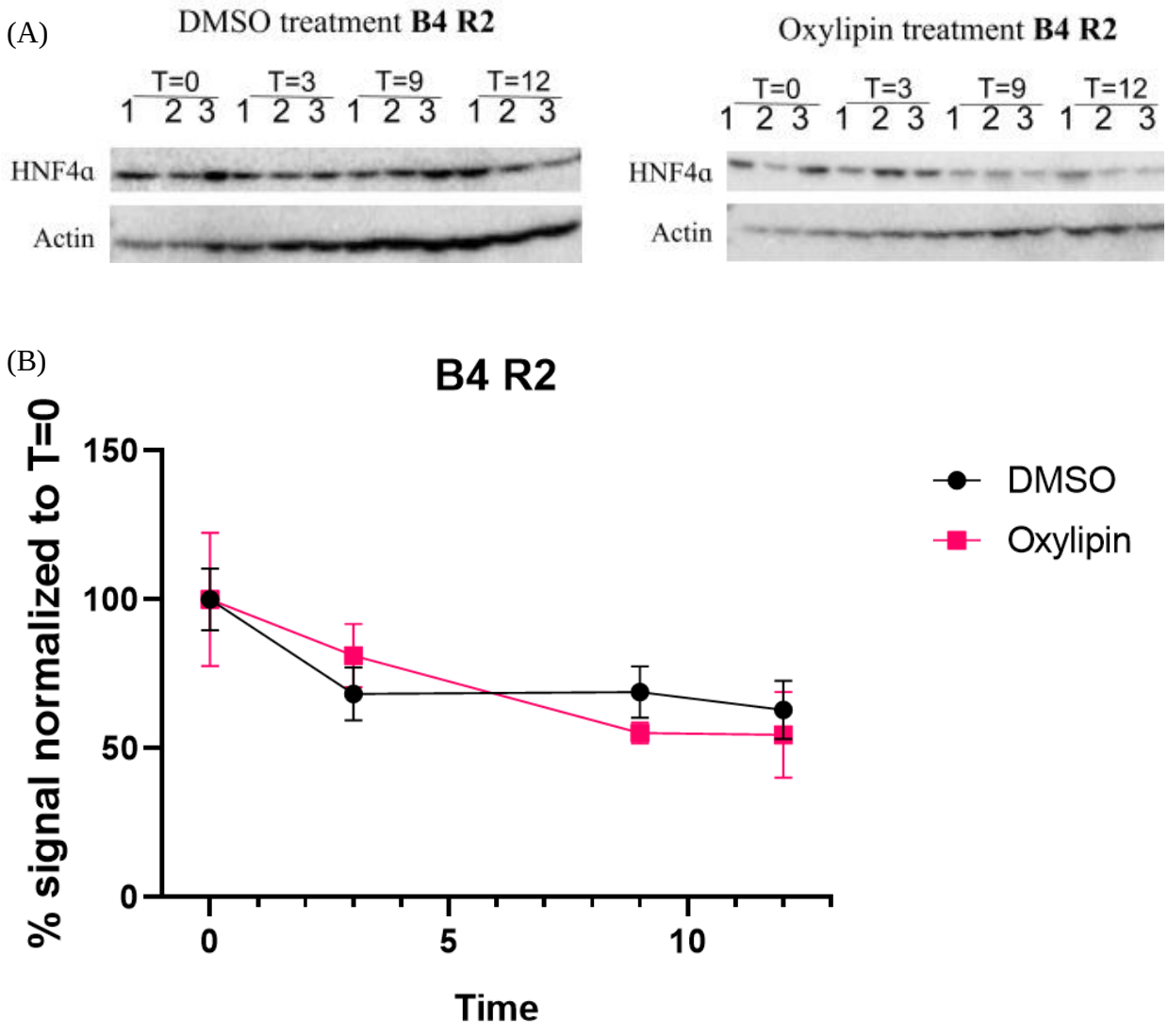


Figure 5 The presence of oxylipins in media does not destabilize HNF4α.

(A) Immunoblot analysis of HNF4α P1/P2 in HCT116 cells induced by doxycycline and treated with DMSO (0.1%) or oxylipins (10 µM). (B) Graph of HNF4α signal normalized to beta-actin,

then to the average value of T=0 bands. The immunoblots shown used samples from experiment number 4 (biological replicate 4, B4); this is the second analysis of those samples, technical replicate 2 (technical replicate 2, R2).

The immunoblots in figure 5 do not show that over 12 hours, the relative levels of HNF4α protein in cells exposed to oxylipins are lower than the levels of HNF4α protein in cells exposed to DMSO, the vehicle control. Multiple comparisons after 2-way ANOVA do not show a significant difference between the two treatments at any time after exposure ($p>0.05$).

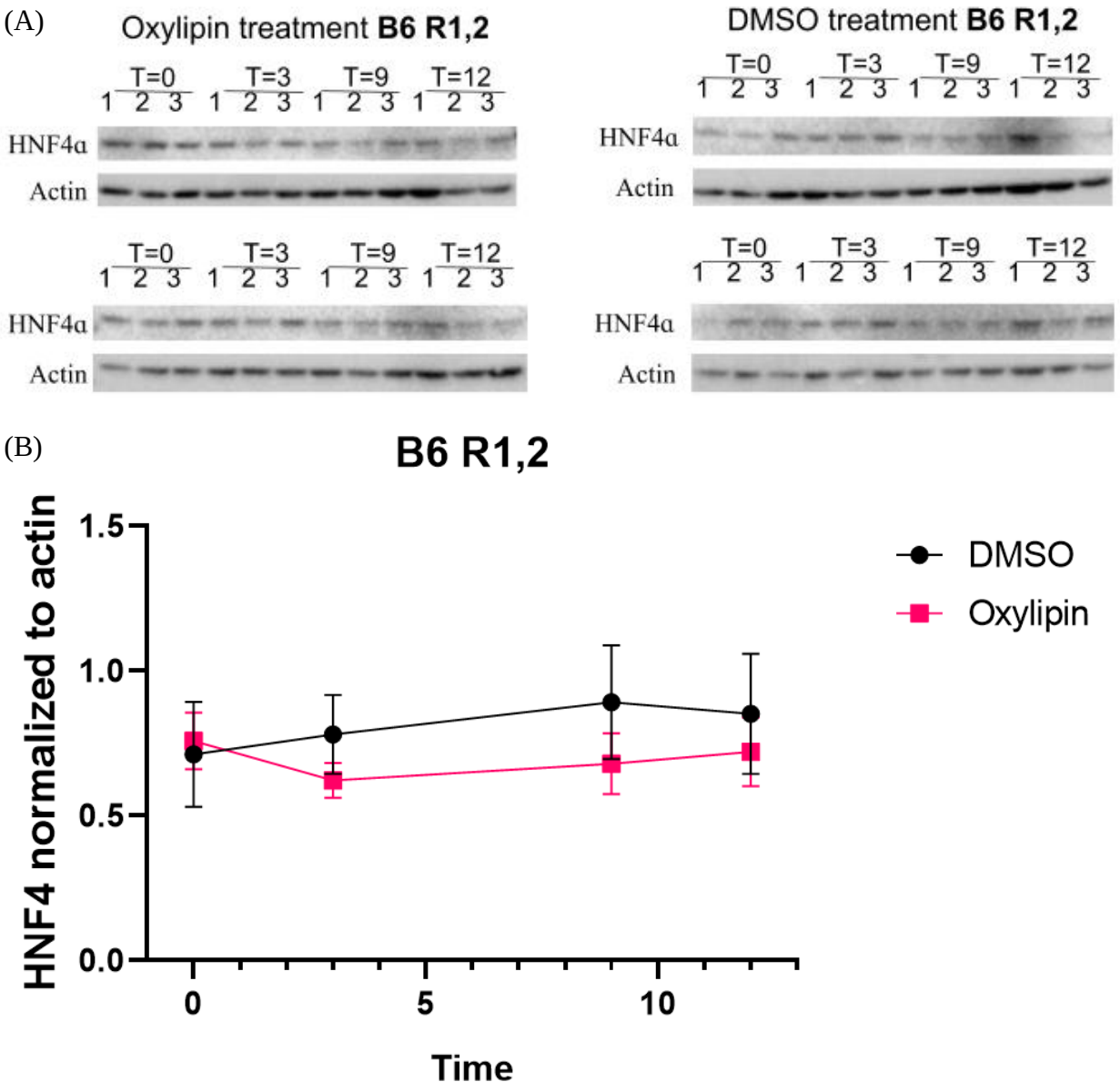
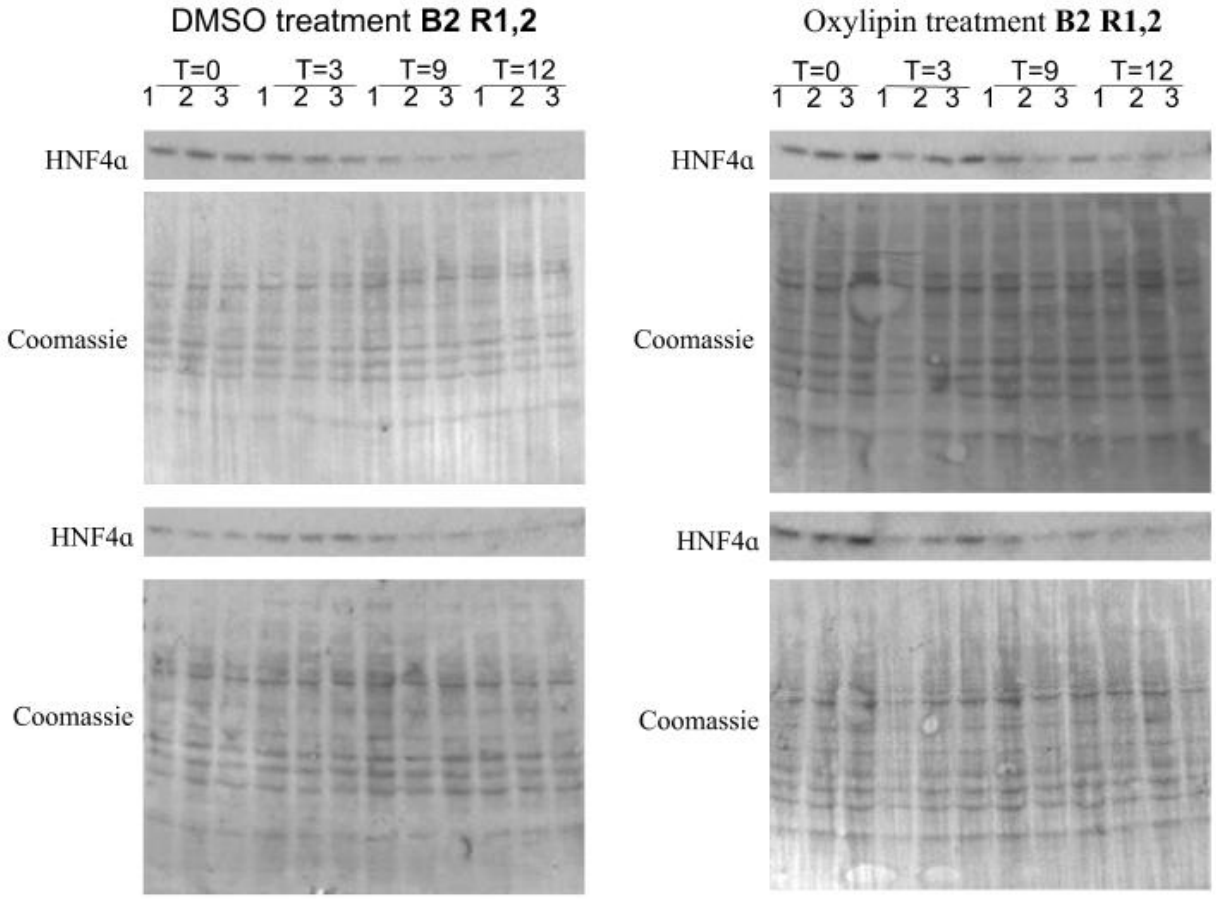


Figure 6 The presence of oxylipins in media does not destabilize HNF4α.

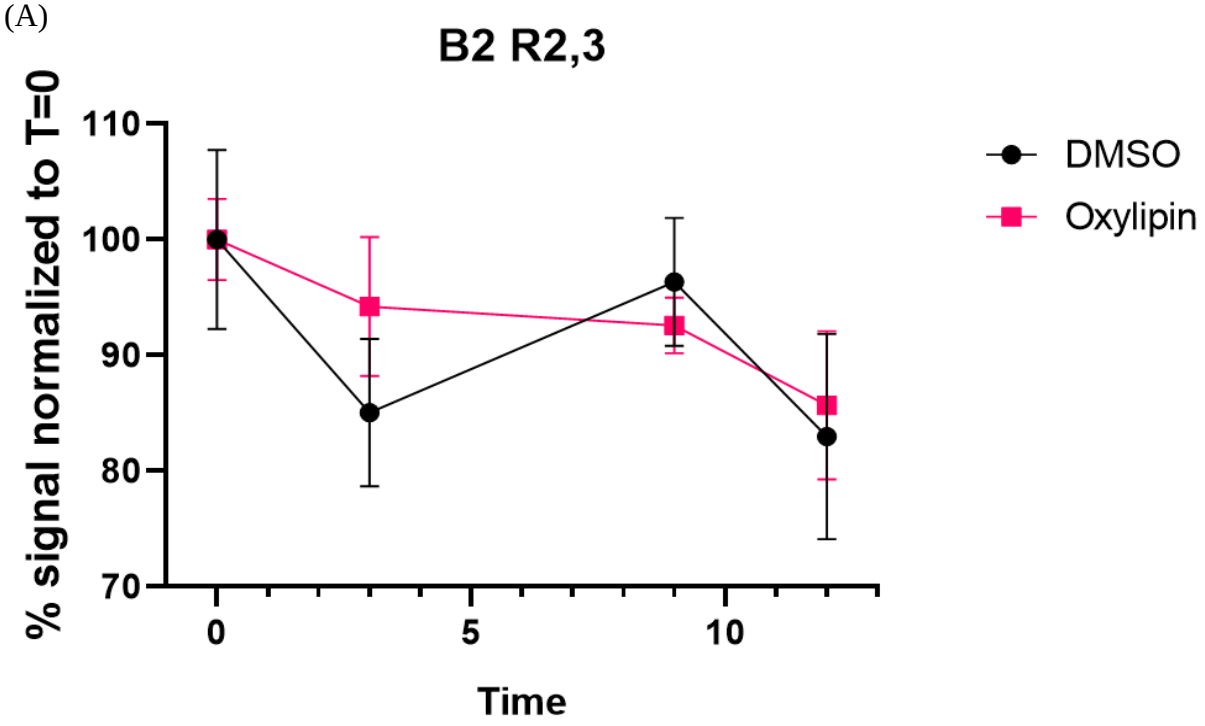
(A) Immunoblot analysis of HNF4α P1/P2 in HCT116 cells induced by doxycycline and treated with DMSO (0.1%) or oxylipins (10 µM). (B) Graph of HNF4α signal normalized to beta-actin. The immunoblots shown used samples from experiment number 6 (biological replicate 6, B6); this is the second analysis of those samples, technical replicates 1 and 2 (technical replicate 1,2, R1,2).

The immunoblots in figure 6 do not show that over 12 hours, the relative levels of HNF4α protein in cells exposed to oxylipins are lower than the levels of HNF4α protein in cells exposed

to DMSO, the vehicle control. Multiple comparisons after 2-way ANOVA do not show a significant difference between the two treatments at any time after exposure ($p>0.05$). However, relative levels of HNF4α protein increases in cells exposed to DMSO while relative levels of HNF4α remain the same in cells exposed to oxylipins. The Y axis in Figure 6 (B) is different than the Y axis in other figures to highlight this increase seen in DMSO treated cells.



(A)



(B)

Figure 7 The presence of oxylipins in media does not destabilize HNF4α.

(A) Immunoblot analysis of HNF4α P1/P2 in HCT116 cells induced by doxycycline and treated with DMSO (0.1%) or oxylipins (10 µM). (B) Graph of HNF4α signal normalized to Coomassie, then to the average value of T=0 bands. The immunoblots shown used samples from experiment number 2 (biological replicate 2, B2); this is the second analysis of those samples, technical replicates 2 and 3 (technical replicate 2,3, R2,3).

The immunoblots in figure 7 do not show that over 12 hours, the relative levels of HNF4α protein in cells exposed to oxylipins are lower than the levels of HNF4α protein in cells exposed to DMSO, the vehicle control. Multiple comparisons after 2-way ANOVA do not show a significant difference between the two treatments at any time after exposure ($p>0.05$).

DISCUSSION

Consumption of soybean oil, which is high in linoleic acid (LA, has increased in recent years both in the United States and world-wide. This increased consumption parallels a rise in obesity in humans (Centers for Disease Control and Prevention, 2018). In mice, soybean oil has been shown to cause obesity (Deol et. al. 2015; Deol et. al. 2017). HNF4α, a nuclear receptor which binds LA, is a master regulator of liver-specific gene expression. In this work we analyzed whether oxylipin metabolites of LA impact HNF4α protein stability in a cell-based system.

Of four comparisons made between the relative HNF4α levels over 12 hours in cells exposed to DMSO or to oxylipins, only one comparison showed a significant difference in the relative HNF4α levels (Figure 4). Another comparison showed that relative levels of HNF4α protein remained the same over 12 hours in cells exposed to oxylipins, while HNF4α levels increased in cells exposed to DMSO (Figure 6). Both comparisons supported our initial hypothesis, that oxylipins destabilize HNF4α protein. However, the other immunoblots imaged (Figures 5 and 7) did not support our initial hypothesis.

The comparisons presented in this study had varying results. Figures 4 and 5 used the same whole cell extracts yet figure 4 showed a significant difference in relative HNF4α levels between both conditions while fire 5 did not. This could be due to the two different methods of normalization utilized (Coomassie and actin staining, respectively) as well as differences in sample preparation for SDS-PAGE through technical errors such as homogenization of the whole cell extracts and pipetting. Additionally, in this study, immunoblotting is utilized to examine and compare the decrease in HNF4α protein levels over time between the two conditions as a result of the inhibition of protein synthesis through cycloheximide and exposure to DMSO or oxylipins. The presence of doxycycline, meant to promote HNF4α protein synthesis through the

inducible Tet-On system, after the addition of cycloheximide, a protein synthesis inhibitor, may obscure these results. More images of the existing blots would need to be collected to normalize all blots to beta-actin and more technical replicates would need to be used to provide controls for SDS-PAGE sample preparation and protein transfer to a PVDF membrane. Overall, the presence of oxylipins in media may impact the levels of HNF4α in the cell, with trends suggesting that oxylipins may induce HNF4α destabilization.

While there is a trend to suggest that the presence of oxylipins in media is sufficient to destabilize HNF4α, there are multiple future directions to pursue to confirm whether this is correct and expand upon our knowledge. The protein samples collected in the experiments shown here only extend to 12 hours past exposure to either the control treatment, DMSO, or oxylipins. These experiments could be redone with additional timepoints past 12 hours to determine whether the difference in the changes in HNF4α levels between both treatments become more pronounced with additional time.

Unpublished data from the Sladek lab indicates that there are lower levels of HNF4α in the hepatocyte nuclei in mice exposed to higher levels of oxylipins. Previously published data (Yuan et. al. 2009) used HCT116 cells infected with a tetracycline-inducible recombinant adenovirus expressing rat HNF4α1. Expression of rat HNF4α1 was induced for 60 hours in the presence of either DMSO or LA, which is structurally similar to oxylipins, and a slight decrease in relative levels of HNF4α in the nuclei of cells exposed to LA was observed. However, the data which suggested that oxylipins may destabilize HNF4α utilized nuclear extracts, while this study examined HNF4α levels in whole cell extracts. The initial hypothesis of this study was that oxylipins destabilized HNF4α, which resulted in the lower levels of HNF4α seen in the nuclei of hepatocytes and HCT116 cells. While destabilization may result in lower levels of HNF4α in the

nucleus, changes in subcellular localization may also produce the same effect. Whole cell extracts would be able to visualize general destabilization of a protein but would not be able to report the localization of a protein in the cell. Future experiments will compare the subcellular localization of HNF4α in cells exposed to DMSO compared to cells exposed to oxylipins dissolved in DMSO.

The possibility that oxylipins may destabilize HNF4α could have a serious impact on dietary recommendations in Western countries. There exist well known instances of altered xenobiotic drug metabolism as a result of diet, such as with St. John's Wort extracts and estradiol (Whitten et. al. 2006). This combination is contraindicated and alters the efficacy of the estradiol, and as a result, health care providers often provide dietary recommendations to avoid undesirable interactions. Because HNF4α regulates the expression of many enzymes responsible for xenobiotic metabolism, particularly CYP enzymes, it is possible that suppression of HNF4α protein and hence function may, over time, lead to impaired xenobiotic metabolism. Should oxylipins be found to suppress HNF4α function, changes to dietary recommendations may be made. Such as suggestions to lower levels of dietary LA for groups in which proper xenobiotic metabolism is crucial, particularly for patients on prescription medications.

The possibility that oxylipins may suppress HNF4α function may be especially important for pregnant women, both during gestation and while breast feeding. Essential fatty acids, such as LA, are transported across the placenta into the fetus (Duttaroy, 2009). It is possible that increased dietary LA for the mother could lead to increased levels of LA and, subsequently, oxylipins in the fetal liver. Suppression of HNF4α function could negatively impact growth and development of the fetus as HNF4α is essential for liver function and development (Hayhurst et, al. 2001) and HNF4α knockout is embryonic lethal in mice (Chen et. al. 1994). Additionally,

composition of breast milk is affected by maternal diet (Friel et. al. 2012), and a maternal diet high in LA could lead to elevated levels of LA in infant livers. Impacted liver development would later impact proper liver function for roles such as glucose homeostasis and drug detoxification.

In summary, the presence of oxylipins in media may impact relative levels HNF4α in whole cell extracts although additional studies are required, especially with respect to subcellular localization. The possibility that oxylipins may impact HNF4α function has many health implications in Western countries, where consumption of soybean oil has increased dramatically and consumption of soybean oil leads to elevated levels of oxylipins in the liver (Deol et. al. 2017).

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