Caffeine increases Nr1i3 expression and potentiates the effects of its ligand, TCPOBOP, in mice liver

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Caffeine is one of the world's most consumed substances. It is present in coffee, green tea and guarana, among others. The xenobiotic-sensing nuclear receptor subfamily 1, group I, member 3 (Nr1i3), also known as the Constitutive Androstane Receptor (Car) is a key regulator of drug metabolism and excretion. No consistent description of caffeine effects on this receptor has been described. Thus, to unravel the effects of caffeine on this receptor, we performed experiments in mice. First, C57Bl/6 mice that were treated daily with caffeine (50 mg/kg) for 15 days presented a slight but significant increase in Nr1i3 and Cyp2b10 gene expression. A second experiment was then performed to verify the effects of caffeine on TCPOBOP (1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-tetrachloro-1,4-*bis*(pyridyloxy) benzene), the most potent agonist known for mice Nr1i3. Interestingly, caffeine potentiated TCPOBOP pleiotropic effects in mice liver, such as hepatomegaly, hepatotoxicity, hepatocyte proliferation and loss of cell-to-cell communication through gap junctions. In addition, caffeine plus TCPOBOP treatment increased liver gene expression of Nr1i3 and Cyp2b10 comparing with only caffeine or TCPOBOP treatments. Together, these results indicate that caffeine increases the expression of Nr1i3 in mice liver, although at this point it is not possible to determine if Nr1i3 directly or indirectly mediates this effect.

Uniterms: Caffeine/effects/experimental study. Androstane constitutive receptor/caffeine effects. 1,4-*bis*-[2-(3,5-dichloropyridiloxy)]benzeno,3,3′,5,5-tetracloro-1,4-*bis*(pyridiloxy)benzene/caffeine effects. Cytochrome p450.

A cafeína é uma das substâncias mais consumidas mundialmente, estando presente no café, chá-verde e guaraná, entre outros. O receptor sensor de xenobióticos Receptor Nuclear subfamília 1, grupo I, membro 3 (Nr1i3, mais conhecido como Androstano Consititutivo - Car) é um regulador chave da biotransformação e excreção de substâncias e nenhuma descrição consistente dos efeitos da cafeína sobre este receptor foi feita. Então, para avaliar os efeitos da cafeína sobre este receptor, realizamos experimentos em camundongos. Primeiramente, camundongos C57/Bl/6 foram tratados diariamente com cafeína (50 mg/kg) por 15 dias e apresentaram um leve, mas significativo, aumento na expressão do Car e do seu gene alvo Cyp2b10. Assim, um segundo experimento foi realizado para verificar os efeitos da cafeína sobre o TCPOBOP (1,4-*bis*-[2-(3,5-dicloropiridiloxi)]benzeno,3,3′,5,5′-tetracloro-1,4-*bis*(piridiloxi)benzeno), o mais potente agonista do Nr1i3 de camundongos conhecido. Interessantemente, a cafeína potencializou os efeitos pleiotrópicos do TCPOBOP no fígado dos camundongos, como hepatomegalia, hepatotoxicidade, proliferação celular e perda da comunicação intercelular por junções do tipo gap. Os camundongos tratados com cafeína e TCPOBOP apresentaram maior expressão gênica de Nr1i3 e Cyp2b10, quando comparados aos camundongos tratados apenas com cafeína ou TCPOBOP. Juntos, nossos resultados indicam que a cafeína aumenta a expressão do receptor CAR em fígados de camundongos C57/Bl/6, porém nesta etapa ainda não é possível afirmar se estes efeitos são direta ou indiretamente mediados pelo Nr1i3.

Unitermos: Cafeína/efeitos/estudo experimental. Receptor constitutivo de androstano/efeitos da cafeína. 1,4-*bis*-[2-(3,5-dicloropiridiloxi)]benzeno,3,3,5,5-tetracloro-1,4-*bis*(piridiloxi)benzeno/efeito da cafeína. Citocromo p450.

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INTRODUCTION

Caffeine is one of the most consumed substances in the world, being present in coffee (*Coffea sp.*), green tea (*Camelia sinensis*), guaraná (*Paullinia cupana*) and also in pharmaceutical formulations (i.e. associated with acetaminophen as in Tylenol®) and soft drinks (Mandel, 2002; Fukumasu *et al.*, 2006). This xanthine alkaloid is best known for its psychoactive stimulatory effects as a consequence of competitive antagonism for the adenosine receptor (Fisone, Borgkvist, Usiello, 2004). After ingestion, caffeine is readily absorbed by the gastrointestinal system, distributed throughout the entire body and quickly metabolized, mostly by the liver, in three different metabolites: paraxanthine, theobromine and theophylline (Lelo *et al.*, 1986). This biotransformation is catalyzed mostly by CYP1A2; however, other cytochrome p450 enzymes are involved, as CYP3A11 and CYP2C9 (Kot, Daniel, 2007). As expected, caffeine treatment induces CYP1A2 expression in liver (Chen *et al.*, 1996; Goasduff *et al.*, 1996) but there is no evidence about the mechanism by which this enzyme expression is increased. Aryl-Hydrocarbon Receptor (AHR) is one of the receptors that regulate CYP1A family expression by binding of its specific ligands (Ma, Lu, 2007). Nonetheless, attempts failed to demonstrate that caffeine-induced CYP1A expression is due to AHR activation (Ayalogu *et al.*, 1995).

Xenobiotic-sensing nuclear receptors such as AHR, the Nuclear Receptor subfamily 1, Group I, member 3 (NR1I3, also known as the Constitutive Androstane Receptor) and the Nuclear Receptor subfamily 1, Group I, member 2 (NR1I2, also known as the Pregnane-X Receptor), have been identified as key regulators in drug metabolism. The mechanism of action behind these effects is the tight transcriptional regulation of several phase I and II drug-metabolizing genes (such as CYP2B, CYP3A, UGT1A1 and GSTA1), as well as drug transporter genes including Mrp2 and Oatp4 (Willson, Kliewer, 2002). Accordingly, NR1I3 has been implicated in the metabolism of several xenobiotics (Wei *et al.*, 2000; Zhang *et al.*, 2002; Xie *et al.*, 2003) as well as endobiotics ((Xie *et al.*, 2003; Guo *et al.*, 2003; Maglich *et al.*, 2004; Saini *et al.*, 2004). The pesticide contaminant 1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is generally considered the most potent mouse Nr1i3 ligand known (Poland *et al.*, 1980). In addition, TCPOBOP administration to mice induces the expression of Cyp2b10 through Nr1i3 activation (Tzameli *et al.*, 2000). We previously showed that a single administration of TCPOBOP to mice induced hepatomegaly doubling liver weight at 72 h (Fukumasu *et*

al., 2010). This effect mainly occurred due to increased cell proliferation (hyperplasia) over hepatocyte hypertrophy after TCPOBOP administration (Fukumasu *et al.*, 2010; Bugyik *et al.*, 2012). In addition, TCPOBOP transiently disrupted intercellular communication by GAP junctions in mouse liver (Fukumasu *et al.*, 2010).

Therefore, we show here that oral (gavage) administration of caffeine to mice for 15 days altered Nr1i3 and Cyp2B10 gene expression in liver. We also further characterized the effects of caffeine administration along with TCPOBOP, since this substance is known as the most potent mouse Nr1i3 ligand.

MATERIAL AND METHODS

Chemicals

Caffeine was obtained from Labsynth (purity of >98.5%; São Paulo, SP, Brazil). TCPOBOP was synthesized as previously described (Dagli *et al.*, 2004) and was kindly provided by Mrs. Croizy (Paris, France). Corn oil was Mazola® (São Paulo, Brazil). Alanine-aminotransferase reagent kit was purchased from CELM (São Paulo, Brazil). The 3´3-diaminobenzidine (DAB), Trizma, Lucifer yellow, rhodamine, formalin, eosin, hematoxylin, and horseradish peroxidase anti-mouse IgG antibodies were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). The Proliferating Cell Nuclear Antigen (PCNA) antibody, skimmed milk and LSAB were from Dako (Glostrup, Denmark). TRIzol, oligoDT primers, superscript II enzyme and Platinum SYBR Green were obtained from Invitrogen (Carlsbad, CA, USA). Assay-on-Demand Gene Expression CAR (Mm004986_m1) and 18s (4319413E) and Taqman Mastermix were from Applied Biosystems (Foster City, CA, USA). Primers for Cyp2b10 and 18s were made by IDT technologies (Coralville, IA, USA).

Animal experiments

Three-month-old female C57Bl/6 mice were bred at the animal facility of the Department of Pathology at the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil, on a 12-hour light/12-hour dark cycle under controlled temperature conditions ($20 \pm 4 \,^{\circ}C$), and relative humidity ($55 \pm 10\%$). One replicate of experiment I was also performed at the Animal Facility of the Department of Basic Sciences at the School of Animal Science and Food Engineering of the University of São Paulo. Conditions at both animal facilities were the same. Mice had ad libitum

access to a standard diet (NUVILAB-CR1®, Nuvital Nutrientes LTDA, São Paulo, Brazil) and filtered water. All procedures using animals were performed following "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised in 1985) and were reviewed and approved by the Bioethics Committee of the SVMAS-USP (process number 953/2006).

The first experiment was performed with 10 females sorted randomly into two groups named CT (for control group) and CAF (for caffeine-treated). Caffeine (50 mg/kg) was diluted in filtered water and administered by gavage once a day for 15 days, while the control group received only filtered water (Figure 1). All animals were weighed every three days during the experiment and were quickly euthanized by deep anesthesia (thiopental 250 mg/kg, I.P., Thipentax® São Paulo, Brazil). Blood was collected from the heart for serum chemistry and livers were harvested, weighed and sampled for histological analysis or snap frozen in liquid nitrogen and conserved at -80 ºC until further analysis. The caffeine dose employed here (50 mg/kg) was considered safe for mice and used previously in other experiments (Chan *et al.*, 2006). No intention to extrapolate this concentration to humans was considered in this experimental work.

The second experiment (Figure 1) was exactly as described above using 62 animals randomly sorted into two groups: CT (for control group) and CAF (for caffeine group). After 15 days of caffeine treatment by gavage, all animals were administered water, corn oil (vehicle) or a single dose of TCPOBOP (6 mg/kg) diluted in corn oil through gavage. They were maintained on caffeine or water treatment until euthanasia for 24, 48 and 72 h (2-3 animals per treatment and time point), following the same procedures described for experiment 1 and published previously by our group (Fukumasu *et al.*, 2010).

Histopathology and serum ALT analysis

Representative slices from each liver lobe were fixed in 10% formalin for 48 hours, dehydrated, processed, embedded in paraffin wax and stained with Hematoxylin and Eosin. Other major organs were also collected for analysis. Biochemical analysis of alanine-aminotransferase was performed accordingly to manufacturer's instructions (CELM, São Paulo, Brazil).

Gene expression analysis by Real-time PCR

Total RNA was isolated from frozen liver tissues with TRIzol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Then, photometrical
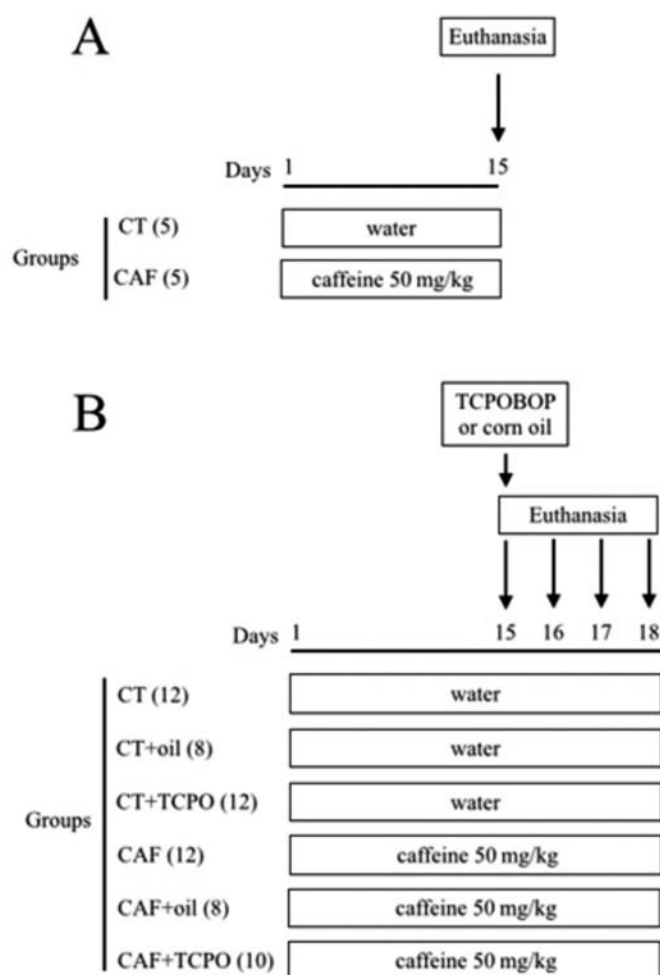

FIGURE 1 - Experimental design. (A) Experiment I that evaluated the effects of caffeine on C57BL/6 female mice for 15 days. (B) Experiment II where caffeine was evaluated in a model of Nr1i3 induction by the ligand TCPOBOP.

quantification was performed (Biophotometer, Eppendorf, Germany) for the 260/280 ratio. Only samples which had 1.7-2.0 and presented good quality after RNA electrophoresis in agarose gel (1.5%, TAE) were used. One µg of total RNA from each sample was treated with DNAse I and reverse transcribed with oligoDT primers and superscript II into cDNA. The cDNAs samples were used for real-timePCR carried out in an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA, USA). For Nr1i3 analysis, Taqman Assay-on-Demand Gene Expression Products (Applied Biosystems) were used as follows: Nr1i3 (Mm004986_m1) and 18s (4319413E). For Cyp2b10 gene expression the SYBR-green method was used (Platinum SYBR Green, Invitrogen), using the 18s ribosomal gene as the endogenous control. Primers were designed (IDT technologies, Coralville, IA, USA) with Primer-3 software (Rozen and Skaletsky, 2000)

and were run in BLAST (Altschul *et al.*, 1990) to verify the absence of local alignments with DNA and other RNA transcript sequences. The following primers were used: Cyp2b10_F: CCTGTGGTTATGCTGTGTGG, Cyp2b10_R: CCACTAAACATTGGG CTTCC, product size 244bp; 18s_F: CCTGCGGCTTAATTTGACTC, 18s_R: CTGTCAATCCTGTCCGTGTC, product size 45bp). Finally, analysis of relative gene expression data was performed according to the $2^{[-\Delta\Delta Ct]}$ method (Livak, Schmittgen, 2001).

Immunohistochemistry for the proliferation cell nuclear antigen (PCNA)

Detection of the Proliferating Cell Nuclear Antigen (PCNA, PC 10, diluted 1:3200) was performed in representative liver sections (5 μm) from animals from experiment 2 as previously described (Fukumasu *et al.*, 2010). Positive PCNA cells have their nucleus colored brown due to DAB deposition; and at least 1000 cells were counted per animal. The proliferation index was obtained by dividing the number of brown nuclear stained cells by the total number of cells counted for each animal and multiplied per 100. Binucleated cells were not considered for this analysis. PCNA is detected in G1, S and G2 cell cycle phases (Foley *et al.*, 1993), which were all considered in this study with no distinction.

Gap Junction Intercellular Communication (GJIC) analysis

Incision Loading Dye Transfer was performed (Sai *et al.*, 2000) with minor modifications (Fukumasu *et al.*, 2010). Areas stained with LY alone or with RhD were detected by fluorescence emission using a microscope (Nikon Eclipse-800, Japan) equipped with an epi-fluorescence unit. The net area stained with LY alone and the length of incision was quantified with an electronic pen and the software calculated automatically (Image Pro-Plus, version 4.5, Media Cybernetics). At least three of the incision sites per specimen were randomly chosen for analysis and the mean value was used as the data from one animal. The distance of dye transfer from the incision line (area/length) was represented as GJIC and values were expressed as a fraction of the control value.

Statistical analysis

Data are presented as mean ± standard deviation. Two-way ANOVA was used for comparisons where two measures were evaluated: body weight and gene expression through time. Bonferroni post-test was used after two-way ANOVA. All other statistical analyses were performed with Mann-Whitney non-parametric test. The software Prism 5 for windows® (GraphPad Software, Inc, USA) was used for all statistical analyses.

RESULTS

Experiment 1

Daily treatment with 50 mg/kg of caffeine for 15 days to female C57Bl/6 mice had no effect on body weight, liver weight and serum ALT (data not shown). Also, no alterations were observed in necropsies or histopathological analysis of organs such as liver, kidneys, stomach, spleen and lungs (data not shown).

We found an increased expression of Nr1i3 (p=0.04, Figure 2) after caffeine treatment. To confirm this finding, we evaluated the level of Nr1i3 specific target Cyp2b10. Interestingly, Cyp2b10 (p= 0.009, Figure 2) presented a significant increment in gene expression after caffeine treatment. Noteworthy was the fact that sole administration of caffeine for 15 days to mice presented no major effect as weight loss or hepatotoxicity but increased Nr1i3 gene expression and Nr1i3 transcriptional function, since increased levels of Cyp2b10 mRNA was demonstrated.
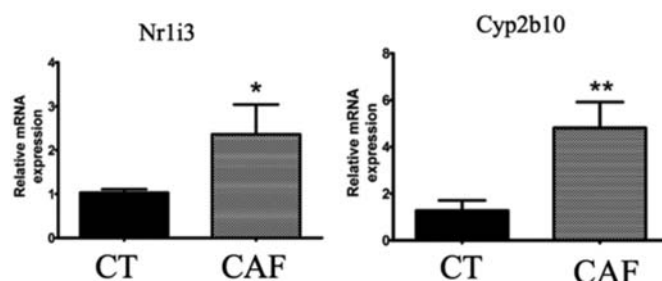

FIGURE 2 - Effects of caffeine in gene expression of Nr1i3 and Cyp2b10. * means P<0.05 between CT and CAF groups; ** P<0.01 between CT and CAF groups.

Experiment 2

We performed the second experiment to verify if caffeine presented an additive effect with the most potent ligand for Nr1i3 in liver, TCPOBOP. After TCPOBOP administration to control and caffeine-treated mice, a significant decrease in body weight was noted in caffeine-treated animals over time (p=0.0027 for treatment, p=0.2917 for time; two way ANOVA, Figure 3). The administration of vehicle (corn-oil) to control- or caffeine-treated mice had no effect or interaction of vehicle with

caffeine on body weight or liver weight (Figure 3). However, caffeine potentiated the effects of TCPOBOP in mice as the TCPOBOP-induced hepatomegaly after 48 h and 72 h ($p < 0.001$ for treatment and time; with a significant interaction of $p = 0.0007$; two way ANOVA, Figure 3) and the TCPOBOP-induced hepatotoxicity after 72 h showed by increased ALT levels in caffeine treated animals ($p < 0.01$, Figure 3). Therefore, since all the effects

FIGURE 3 – Results from experiment II. (A) Body weight of CT or CAF-treated animals for 15 days and then administered by gavage water, corn-oil (vehicle for TCPOBOP) or TCPOBOP. (B) Relative liver weight of CT and CAF-treated mice after TCPOBOP administration (time 0h – day 15). (C) Alanine aminotransferase in sera of CT and CAF treated mice after TCPOBOP administration. * <0.05 and ** $< p.001$.

were seen 48 or 72 h after TCPOBOP administration, we chose to evaluate the cellular and molecular effects of caffeine plus TCPOBOP at 24 h to determine the priming effect of caffeine on TCPOBOP pleiotropic effects.

Twenty-four hours after TCPOBOP administration, caffeine potentiated the TCPOBOP effects shown by increased liver cell hyperplasia ($p = 0.0273$; Fig. 4) and altered cell-to-cell communication by gap junctions ($p < 0.05$, Fig. 4). We also checked if caffeine could have any effects on TCPOBOP-induced gene expression of Nr1i3 and Cyp2b10. First we showed that TCPOBOP increased Nr1i3 and Cyp2b10 gene expression after 24 h of administration (CAR: 1.29 fold, $p = 0.0374$ and Cyp2B10: 54.1 fold, $p < 0.0001$; Figure 4) as did caffeine (Figure 2). Twenty-four hours after TCPOBOP administration, caffeine pre-treatment potentiated the Nr1i3 and Cyp2b10 gene expression after 24 h of TCPOBOP administration by 46% ($p = 0.0055$) and 94% ($p = 0.0444$), respectively, in comparison with TCPOBOP-only treated animals at this time point. Hence, in this second experiment we demonstrated that caffeine potentiated the effects of TCPOBOP in mice liver as shown by macroscopic, microscopic and molecular analysis.
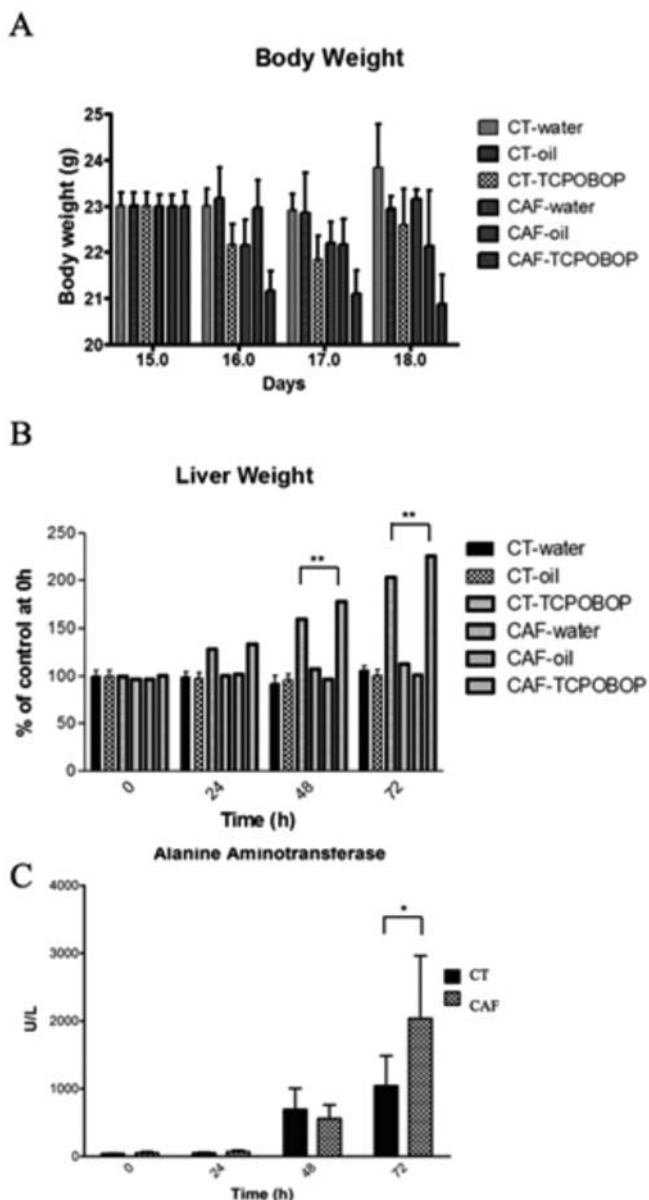
DISCUSSION

In this study, we showed that oral administration of 50 mg/kg of caffeine for 15 days to mice increased gene expression of Nr1i3 and one of its transcriptional targets (Cyp2b10) in the liver. Also, we demonstrated that caffeine potentiated the pleiotropic effects of TCPOBOP in mice liver, a substance known as the most potent mouse Nr1i3 ligand (Poland *et al.*, 1980). These experiments demonstrated that caffeine administration to C57/Bl female mice altered the Nr1i3 function in the liver; however, at this point we are not able to affirm if this effect is direct to Nr1i3 or indirect.

Caffeine, one of the world's most consumed substances, deserves attention regarding its effects on xenobiotic nuclear receptors due to their importance in drug metabolism and biotransformation. As an example, Nr1i3 was shown to play a key role in acetaminophen (APAP) metabolism and hepatotoxicity (Zhang *et al.*, 2002). It is known that APAP toxicity is by far the most common cause of acute liver failure in United States (Larson *et al.*, 2005). The combination of APAP and caffeine is used worldwide to treat pain from several conditions such as headaches, colds, fever and others in several commercially available formulations. It has long been demonstrated that caffeine enhances the biotransformation of APAP mostly due to an effect of
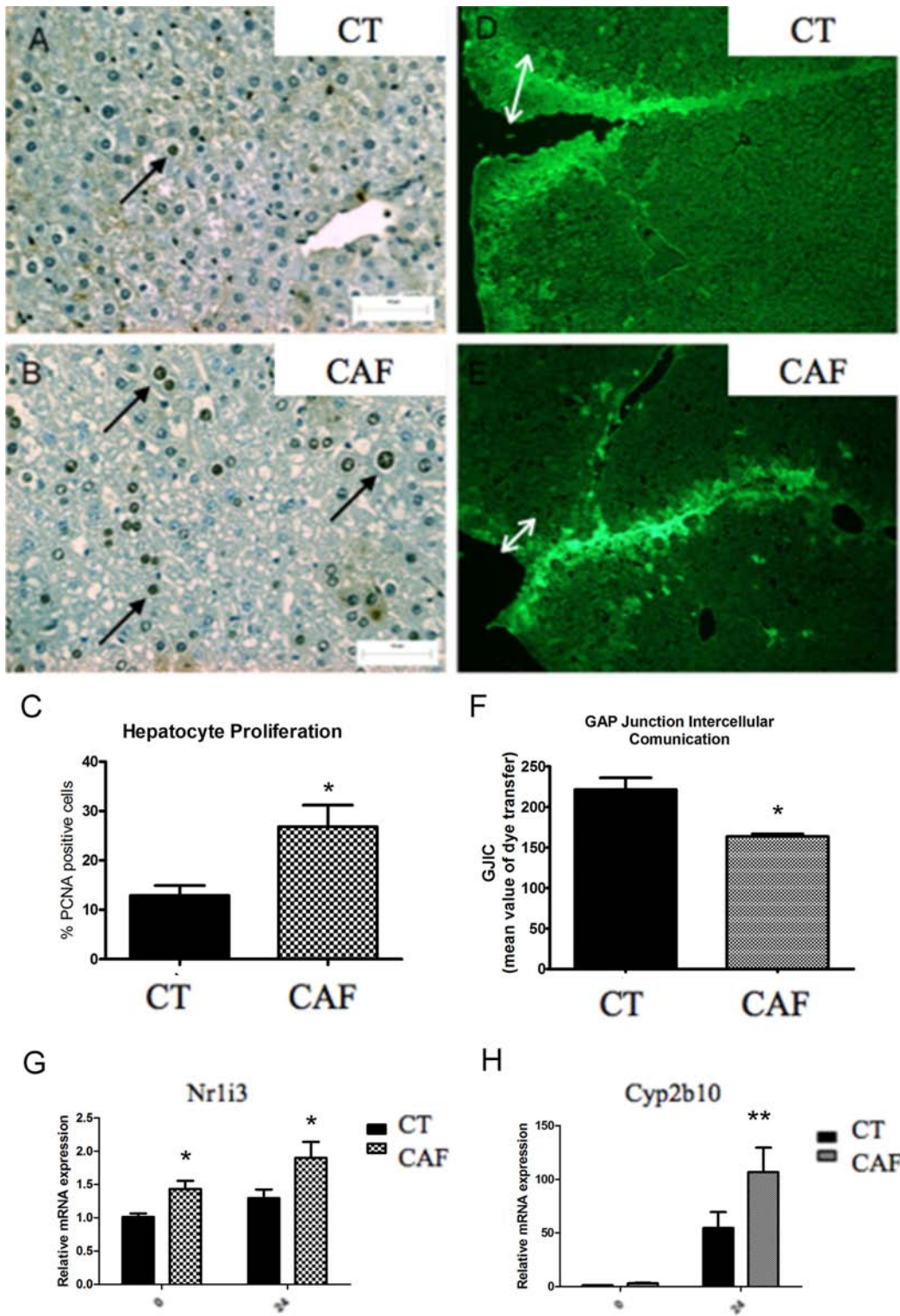
FIGURE 4 – Pleiotropic effects of caffeine treatment after 24 h of TCPOBOP administration. (A, B and C) Representative images and quantification of immunohistochemistry for PCNA after 24 h of TCPOBOP administration between CT and CAF groups. The control samples in this analysis were from animals treated with water and TCPOBOP. (D, E and F) Representative images and quantification of Gap Junctional Intercellular Communication assay after 24 h of TCPOBOP administration between CT and CAF groups. The control samples in this analysis were from animals treated with water and TCPOBOP. (G and H) Expression of Nr1i3 and Cyp2b10 mRNA. * means P<0.05 between CT and CAF groups. Black arrows indicate PCNA positive nuclei. White lines indicated the Lucifer yellow dye transfer.

caffeine on the substrate-enzyme complex rather than an increase in the direct effect of caffeine on APAP binding to cytochrome p450 (Jaw, Jeffery, 1993). In addition, some reports have already demonstrated that prior treatment with caffeine altered APAP metabolism and hence increased its hepatotoxicity in rodents (Sato, Izumi, 1989; Jaw, Jeffery, 1993). When caffeine is used as a pretreatment, it induces the microsomal mixed function oxidase system, leading to a more rapid rate of formation of the hepatotoxic arylating APAP biotransformation product (Gale *et al.*, 1986). Therefore, shedding light on the possible modulation of liver p450 enzymes by caffeine through xenobiotic nuclear receptors is important.

Although we used a dose of caffeine (50 mg/kg) which could be considered elevated in this experiment, mice did not present any toxic effects since no changes in body or liver weight, serum ALT or histopathology of liver and other major organs were noted.   These results are in agreement with a study conducted by the National Toxicology Program (National-Toxicology-Program, 1984) in which caffeine administration to female mice (CD-1 background) showed no effects in body or organ weight even in a high dose such as 93 mg/kg. One alteration noted in our first experiment was the increased gene expression of Nr1i3 and its transcriptional target Cyp2b10, which led us to perform the second experiment where we evaluated the effects of caffeine on TCPOBOP-treated mice.

We previously showed that TCPOBOP under the same protocol used here induced hepatomegaly, hepatotoxicity and loss of cell-to-cell communication through Gap Junctions (Fukumasu *et al.*, 2010). The most prominent effect of TCPOBOP on mice liver was hepatomegaly, as the liver doubled its weight after 72 h due mostly to increased hepatocyte proliferation over hypertrophy. In addition, this effect in cell proliferation could be responsible, in part, for the alterations in gap junction intercellular communication since an inverse association between these two cell processes is well known (Yamasaki *et al.*, 1999).

This mitogenic effect of TCPOBOP in liver cells was recently shown to be a possible alternative for the rescue of regenerative response in cirrhotic livers (Bugyik *et al.*, 2012). However, attention should be given before using Nr1i3 ligands for any therapeutic purposes since we showed here that caffeine, a substance consumed worldwide, potentiated some effects of the Nr1i3 ligand in mice. It could be possible that other commonly consumed substances could modify Nr1i3 ligand effects. In fact, substances present in garlic (Sueyoshi *et al.*, 2011) and

Yin zhi huang, a traditional Chinese herbal extract with *Artemisia capilaris*) (Huang *et al.*, 2004), have been shown as activators of Nr1i3.

CONCLUSION

To the best of our knowledge, this is the first demonstration that caffeine increases Nr1i3 gene expression and potentiated TCPOBOP, the most potent known Nr1i3 ligand in mice. Future studies should be performed to determine if these effects of caffeine directly or indirectly modulate Nr1i3.

ACKNOWLEDGMENTS

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