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Posttranslational regulation of CYP2J2 by nitric oxide

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ABSTRACT

Nitric oxide (NO) is an essential signaling molecule in the body, regulating numerous biological processes. Beside its physiological roles, NO affects drug metabolism by modulating the activity and/or expression of cytochrome P450 enzymes. Previously, our lab showed that NO generation caused by inflammatory stimuli results in CYP2B6 degradation via the ubiquitin-proteasome pathway. In the current study, we tested the NO-mediated regulation of CYP2J2 that metabolizes arachidonic acids to bioactive epoxyeicosatrienoic acids, as well as therapeutic drugs such as astemizole and ebastine. To investigate the effects of NO on CYP2J2 expression and activity, Huh7 cells stably transduced with CYP2J2 with a C-terminal V5 tag were treated with dipropylenetriamine-NONOate (DPTA), a NO donor. The level of CYP2J2 proteins were decreased in a time- and concentration-dependent manner, and the activity was also rapidly inhibited. However, mRNA expression was not altered and the protein synthesis inhibitor cycloheximide did not attenuate DPTA-mediated downregulation of CYP2J2. Removal of DPTA from the culture media quickly restored the activity of remaining CYP2J2, and no further CYP2J2 degradation occurred. To determine the mechanism of CYP2J2 down-regulation by NO, cells were treated with DPTA in the presence or absence of protease inhibitors including proteasomal, lysosomal and calpain inhibitors. Remarkably, the down-regulation of CYP2J2 by NO was attenuated by calpeptin, a calpain inhibitor. However, other calpain inhibitors or calcium chelator show no inhibitory effects on the degradation. The proteasome inhibitor bortezomib showed small but significant restoration of CYP2J2 levels although stimulated ubiquitination of CYP2J2 was not detected. In conclusion, these data suggest that NO regulates CYP2J2 posttranslationally and NO-evoked CYP2J2 degradation undergoes ubiquitin-independent proteasomal degradation pathway unlike CYP2B6.

1. Introduction

Nitric oxide (NO) is one of the key signaling molecules in the body and regulates various physiological functions including cardiovascular, immune, and nervous systems as well as pathological processes [1]. Endogenous NO is generated by nitric oxide synthase (NOS) and higher levels of NO are generated during infection and inflammation by inducible NOS (NOS2). Besides the activation of immune systems, clinical drugs also can elevate NO levels by directly releasing NO, inducing the expression of NOS2 or increasing NO bioavailability [2–4]. In the classical mechanism of NO signaling, NO promotes the generation of cyclic guanosine monophosphate (cGMP) by activating soluble guanylate cyclase, which controls diverse biological functions and gene expressions [5]. On the other hand, as a diffusible signaling molecule across the cellular membrane, NO can also directly interact with a variety of proteins, affecting protein turnover, activity, and translocation [6–8]. Cytochrome P450 (P450) enzymes are one family of proteins affected by NO. For example, Khatsenko et al. [9] reported that the inhibition of P450 activity and decreased P450 protein contents by treatment with lipopolysaccharide were mediated by overproduced NO in phenobarbital-treated rats [9]. The study also implicated direct binding of NO to heme within P450. Apart from the heme nitrosylation, in vitro cysteine nitrosylation of several P450s and tyrosine nitration of CYP2E1 and CYP3A4 have been reported [10–12].

In our previous studies, we showed that NO mediated the down-regulation of several P450 enzymes including CYP2C22, CYP2B1, CYP2B6, and CYP51A1 [10,13–15]. In common, the down-regulation of mRNA of the identified P450 enzymes was much slower than the rate of

Abbreviations: 3-MA, 3-methyladenine; AA, arachidonic acid; ASC, ascorbate; Bort, bortezomib; BST, biotin-switch technique; Calp, calpeptin; CHX, cycloheximide; Dan, danazol; DMEM, Dulbecco's modified Eagle Medium; DPTA, dipropylenetriamine NONOate; EETs, epoxyeicosatrienoic acids; EST, (2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; GSNO, S-nitrosoglutathione; HA-Ub, hemagglutinin-ubiquitin; HMM, higher molecular mass; L-NAME, N^{ω}-Nitro-L-arginine methyl ester hydrochloride; MG132, Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; MMTS, methyl methanethiosulfonate; NO, nitric oxide; NOS2, inducible nitric oxide synthase; NOx, nitrate + nitrite; P450, cytochrome P450

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protein degradation. However, interestingly the NO-mediated proteolytic mechanisms were different from each other. For instance, CYP2B6 was polyubiquitinated in response to NO and the down-regulation was blocked by a proteasome inhibitor bortezomib [14]. However, CYP2C22 degradation was not rescued by inhibition of proteasomal, lysosomal or calpain pathways, suggesting the participation of novel proteolytic systems [13].

Unlike those mentioned above, not all P450 proteins are sensitive to down-regulation by NO (eg. CYP2C11 and CYP3A4) [14,16,17]. Therefore, in the current study we tested the effect of NO on the expression and activity of CYP2J2. As the only member of the human CYP2J subfamily, CYP2J2 is one of the most important CYP epoxygenases in the metabolism of arachidonic acid (AA). The epoxygenation of AA generates 5, 6-, 8, 9-, 11, 12- and 14, 15-epoxyeicosatrienoic acids (EETs), which mediate diverse biological functions including anti-inflammation, vasodilation and tissue protection [18–20]. CYP2J2 is broadly expressed in various tissues including heart, lung, kidney, intestine, and liver. Among those tissues, CYP2J2 is predominantly expressed in the cardiovascular system and shows cardiovascular protective effects. For example, CYP2J2 cardiomyocyte-specific CYP2J2 expression attenuated angiotensin II-induced cardiac fibrosis, cardiac hypertrophy and dysfunctions in mice, which was mimicked by 11, 12- and 14, 15-EETs administrations [21,22]. Additionally, the pathological role of CYP2J2 has been reported in tumor growth and metastasis [23–25].

Besides these pathological and physiological roles, CYP2J2 is also responsible for metabolism of therapeutic agents such as terfenadine, astemizole, ebastine, and cyclosporine [26]. Especially, CYP2J2 contributes to the first-pass metabolism of the antihistamines astemizole and ebastine in the intestine [27,28]. Because of the influence of CYP2J2 in drug metabolism, the latest guidance from the US Food and Drug Administration (FDA) suggests that CYP2J2 should be considered in drug metabolism studies of new drugs (US Department of Health and Human Services, 2017) [29].

In this work, we detected down-regulation of CYP2J2 proteins and inhibition of the enzyme activity by a NO donor, dipropylenetriamine NONOate (DPTA) in a time- and dose-dependent manner. Interestingly, the removal of DPTA quickly restored the activity of CYP2J2 and no further degradation of CYP2J2 occurred. In addition, the proteasome inhibitor bortezomib attenuated CYP2J2 degradation by DPTA although the inhibitor did not fully block the down-regulation.

2. Materials and methods

2.1. Reagents

Fetal Bovine Serum was purchased from Atlanta Biologicals (Flowery Branch, GA). Dulbecco's Modification of Eagle's Medium (DMEM) was from Corning (Corning, NY). Bortezomib and calpeptin were obtained from LC laboratories (Woburn, MA) and Calbiochem (San Diego, CA), respectively. Dipropylenetriamine NONOate (DPTA) and S-nitrosoglutathione (GSNO) were from Cayman Chemicals (Ann Arbor, MI). CriterionTM TGXTM Precast Gels (8–16%) were obtained from Bio-Rad Laboratories (Hercules, CA). Lactate dehydrogenase, N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), cycloheximide, danazole, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibodies to the V5-peptide (catalog # V8012), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, catalog # MAB374) and actin (catalog # GTX109639) were purchased from Sigma-Aldrich (St. Louis, MO), Millipore (Billerica, MA), and GeneTex (Irvine, CA), respectively. The monoclonal CYP2J2 antibody was obtained from Abnova (Taipei, Taiwan, catalog # H00001573-M01). IRDye® 680RD Goat anti-Rabbit IgG and IRDye® 800CW Goat anti-Mouse IgG were from LI-COR Biosciences (Lincoln, NE).

2.2. Cell culture

HeLa and Huh7 cell lines were obtained from the American Type Culture Collection and the laboratory of Dr. Arash Grakoui of Emory University, respectively. Each cell line was verified by the Emory Integrated Genomics Core facility. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in humidified 5% CO_2 incubator at 37 °C.

2.3. Lentiviral construction

A pENTR vector containing human CYP2J2 was obtained from the Arizona State/DNASU plasmid repository (https://dnasu.org/DNASU/Home.do). To add the V5 tag, CYP2J2 was subsequently cloned into pLX304 vector by Gateway LR reaction (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. To generate viruses containing CYP2J2v5, human embryonic kidney HEK293T cells were transfected with pLX304-2J2v5 and a second generation lentiviral packing system consisting of pMD2.G and psPAX2 (gifts from Didier Trono; Addgene (Cambridge, MA) plasmids # 12259 and # 12260, respectively) according to the virus production protocol from Addgene (https://www.addgene.org/tools/protocols/plko/). At 48 h and 72 h after transfection, virus-containing supernatants were collected and passed through a 0.45 μm filter. Huh7 cells were infected with the virus as previously described [14] and then selected with 5 μg/ml blasticidin for 10 days. To test effects of endogenously generated NO on P450 regulation, HeLa cells expressing pLIX-hNOS2 under control of tetracycline were generated using a previously employed method [14].

2.4. Real-time PCR

Total RNA was extracted with TRIzol reagent and a Direct-zol RNA Miniprep Kit (Zymo Research; Irvine, CA) according to the manufacturer's instructions. cDNA was then synthesized from 2 μg of RNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Grand Island, NY). Real-time PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) using an ABI 7300 real-time PCR system. CYP2J2 mRNA was normalized with GAPDH mRNA (CYP2J2-CTCAATAGGAA AGCGGGCAT and AGCTTCTCATTGTTTGGGGG; GAPDH- ATCTTCCA GGAGCGAGATCC and AGGAGGCATTGCTGATGATC). Analysis of real-time PCR was carried out by the ΔΔCt method.

2.5. Nitric oxide assay

NO production from HeLa cells containing pLIX-hNOS2 was determined by the levels of nitrite/nitrate (NOx) in the culture media using the Griess reaction [30]. Fifty μl of cell culture media was incubated with the equal volume of 0.2 M potassium phosphate buffer (pH 7.4) containing 200 μM NADPH, 20 μM FAD and 0.2 U/ml of nitrate reductase for 1 h at room temperature. Following the incubation, 11 μl of mixture of lactate dehydrogenase (1500 U/ml) and 0.1 M pyruvic acid (1:10) was added into the solution and incubated at 37 °C for 30 min. Lastly, Griess reagent (equal volumes of 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine) was added to the solution and absorbance was measured at 550 nm.

2.6. Immunoblot analysis

After treatments, cells were harvested with cell lysis buffer containing 50 mM Tris-Cl, pH 7.5, 0.1% SDS, 1% NP-40, 1 mM EDTA, and a protease inhibitor cocktail (Sigma-Aldrich, catalog # P8340) at a dilution of 1:200. Cell lysates were centrifuged at 15,000 ×*g* for 10 min and the supernatants were collected. Protein concentration was determined with a bicinchoninic acid assay kit (Thermo Fisher Scientific, Grand Island, NY). Protein samples were separated by 8–16% SDS-

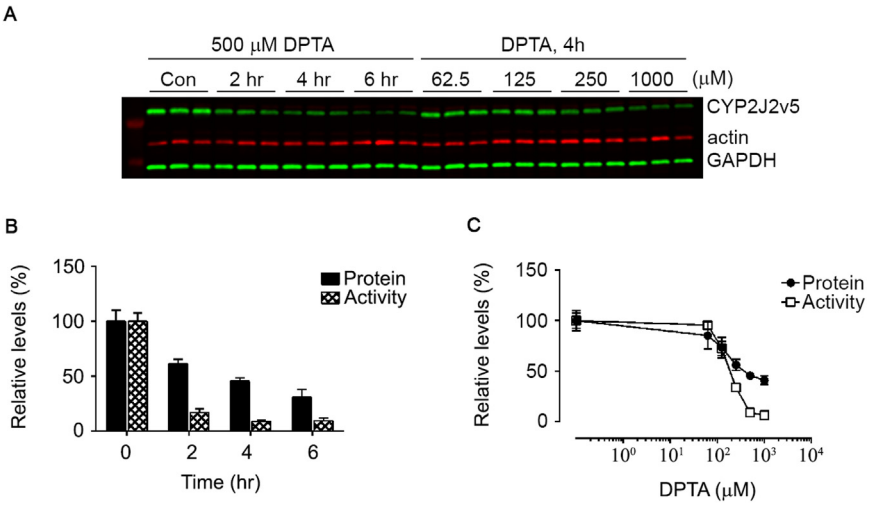

Fig. 1. Down-regulation of CYP2J2 by nitric oxide. Huh7 cells transduced with pLX304-CYP2J2v5 were treated with 500 μM DPTA for different time periods or with different concentrations of DPTA for 4 h. Twenty minutes before harvesting cells, 30 μM Luciferin-2J2/4F12, a CYP2J2 substrate was added to each well to measure the activity of CYP2J2. Produced luciferin in collected culture media was detected as luminescence by adding Luciferin detection reagent from Promega. Cell lysates were subjected to SDS-PAGE and the expression levels of CYP2J2 were determined with an antibody to the V5 tag by Western blotting (A). Fluorescent intensity was quantified using Odyssey FC imaging system and the relative content by time (B) and concentration (C) was plotted as the means ± SD of three independent experiments. Activity was plotted from a single experiment.

PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). To detect V5-tagged CYP2J2, the membranes were probed with the anti-V5 antibody at a dilution of 1:10,000. Actin (1:5,000) and GAPDH (1:10,000) antibodies were added to detect loading controls. IRDye-labeled anti-rabbit or anti-mouse polyclonal secondary antibodies (LI-COR) were used at 1:10,000 and IR fluorescence was detected with an Odyssey® Fc Imaging System (LI-COR Biosciences, Lincoln, NE). Fluorescence intensity was measured using LI-COR Image Studio™ software. Immunoblot data from multiple repeated experiments were combined and normalized as described previously [14].

2.7. Cell-based CYP2J2 activity

CYP2J2 activity was measured with Luciferin-2J2/4F12 (Promega, P1671), a luminogenic CYP2J2 substrate. Cells were grown in 24 well plates and after NO donor treatments, 30 μM substrate was added into culture media. Cells were incubated with the substrate for 20 min and 25 μl of the media was then transferred to a 96 well plate. Equal volumes of Luciferin detection reagent (Promega, V8931) were added and incubated for 20 min at room temperature. Luminescence was analyzed by the FLUOstar OMEGA microplate reader (BMG LabTech, Cary, NC).

2.8. Detection of S-nitrosylated CYP2J2 by biotin-switch technique

S-nitrosylation of CYP2J2 was detected in NO donor-treated Huh7 microsomes using the biotin-switch technique (BST) described by Forrester et al. [31]. To isolate microsomes, Huh7 cells expressing CYP2J2v5 were trypsinized and centrifuged at 200 × *g* for 3 min. Pellets were washed twice with cold PBS and resuspended in 100 mM potassium phosphate buffer containing protease inhibitor cocktail. The resuspended pellets were incubated on ice for 10 min and then sonicated for 10 s at a power setting of 15. Cells were centrifuged at 12,000 × *g* for 15 min and the supernatant was immediately centrifuged using a Beckman type 70 Ti rotor at 45,000 rpm (150,000 × *g*) for 1 h. Pellets were resuspended in microsome storage buffer (buffer composition). Protein concentration was determined with a BCA kit and 200 μg of microsomes were treated with 100 μM GSNO, 100 μM DPTA or 100 μM glutathione (GSH) for 30 min at 37 °C, followed by BST by which S-nitrosylated cysteine residues are labeled with biotin. Briefly, free thiols were blocked by 0.1% methyl methanethiosulfonate (MMTS) in the presence of 2.5% SDS at 50 °C for 20 min. After acetone precipitation, samples were incubated with 20 mM sodium ascorbate and 0.25 mg/ml EZ-Link HPDP-biotin (Thermo Fisher Scientific) at room temperature in the dark for 1 h. Samples were precipitated with acetone to remove residual biotin-HPDP and then biotin-labeled proteins in the resuspended pellets were pulled down with neutravidin agarose resin (Thermo Fisher Scientific).

2.9. Statistical analyses

Data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. Differences between groups were calculated by one-way ANOVA followed by an appropriate post-hoc test using GraphPad Prism software (La Jolla, CA). $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Down-regulation of CYP2J2 by DPTA in a time- and dose-dependent manner

To determine whether nitric oxide could regulate CYP2J2 protein expression, Huh7 cells expressing V5-tagged CYP2J2 were treated with 500 μM of the NO donor DPTA for different time periods, resulting in a decrease of CYP2J2 protein levels to 61%, 46% and 31% of control at 2, 4 and 6 h of treatment, respectively (Fig. 1A, B). In addition CYP2J2 proteins were reduced in a dose-dependent manner, examined with a range of 0–1000 μM DPTA at the 4 h time point (Fig. 1A, C). After indicated time points, CYP2J2 activity was also measured using a luminogenic 2J2 substrate. Interestingly, the activity of CYP2J2 was more quickly and greatly decreased by DPTA treatment. As seen in Fig. 1B, DPTA treatment inhibited CYP2J2 activity to approximately 17% of control within 2 h while 61% of the proteins remained. Additionally, to examine if the V5 tag affects the response to NO, the same experiment was conducted with Huh7 cells transduced with untagged-CYP2J2. Like the ones with CYP2J2v5, wild-type CYP2J2 proteins were similarly down-regulated in a time- and dose-dependent manner (Suppl Fig. 1.)

3.2. No effect of DPTA on transcription and translation of CYP2J2

To elucidate the mechanisms of DPTA-mediated CYP2J2 down-regulation, mRNA levels were measured by quantitative rtPCR. Huh7 cells treated with 500 μM DPTA for 0, 2, 4 and 6 h showed no significant changes in CYP2J2 mRNA levels among different time points (Fig. 2). In addition, the contribution of de novo protein synthesis to the down-regulation of CYP2J2 was tested by incubating cells with DPTA in the presence of the protein synthesis inhibitor cycloheximide (CHX). The rate and extent of down-regulation with CHX were very similar to those in only DPTA-treated samples (Fig. 3, compare with Fig. 1B). These data showing no effects of NO on mRNA expression and new protein synthesis of CYP2J2 clearly indicate that protein degradation is

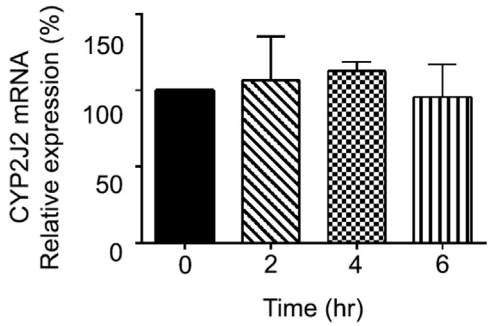

Fig. 2. Effect of nitric oxide on CYP2J2 mRNA expression. Cells were incubated with 500 μM DPTA for 0, 2, 4 and 6 h. After the indicated time period, total RNA was extracted and reverse transcribed. The level of CYP2J2 mRNA was determined by RT-PCR and normalized by GAPDH. Values represent the mean ± SD of values from three independent experiments. Differences between 0 h and each time point group were analyzed by one-way ANOVA and Dunnett's test. No statistical significance was detected.

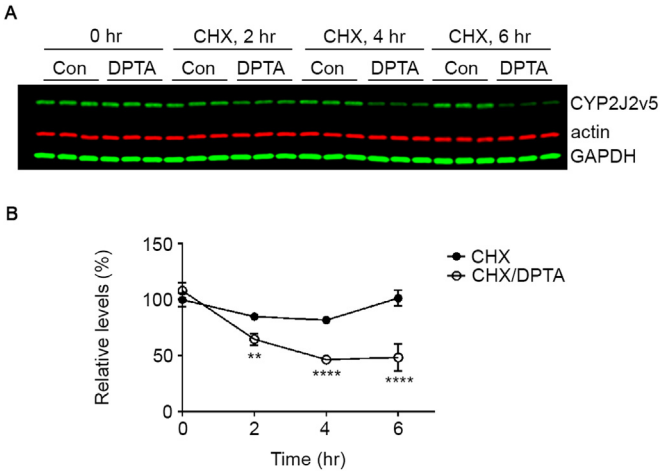

Fig. 3. CYP2J2 down-regulation by nitric oxide in the presence of cycloheximide. Huh7 cells were treated with 500 μM DPTA in the presence or absence of 10 μg/ml of the protein synthesis inhibitor cycloheximide (CHX) for indicated time periods. Protein levels of CYP2J2 were analyzed by Western blotting (A) and relative levels of the quantified fluorescent intensity were plotted with the means ± SD of three independent experiments (B). $^{**}p < 0.01$; $^{****}p < 0.0001$ versus control (Con) cells at same time point; two-way ANOVA with Sidak's correction.

the main mechanism of CYP2J2 down-regulation by NO.

3.3. Down-regulation of CYP2J2 by cellular nitric oxide

Since NO donors have different half-lives and NO-releasing mechanisms, the effect of endogenously- generated NO in CYP2J2 regulation was tested to confirm the NO-mediated CYP2J2 degradation. For the experiment, human NOS2 under control of the tetracycline regulator (TetR) protein was stably transduced into a HeLa cell line. As seen in Fig. 4, doxycycline (Dox) treatment significantly elevated NO production, resulting in a decrease in CYP2J2 expression. The reduced enzyme levels and induced NO generation were blocked by the iNOS inhibitor L-NAME (LN).

3.4. Reversible NO modification

To examine whether the effect of NO on the activity of CYP2J2 is reversible, CYP2J2 activity was measured with or without replacement of fresh media following DPTA treatment for the indicated time points. Since the activity of CYP2J2 was quickly inhibited within 2 h as seen in

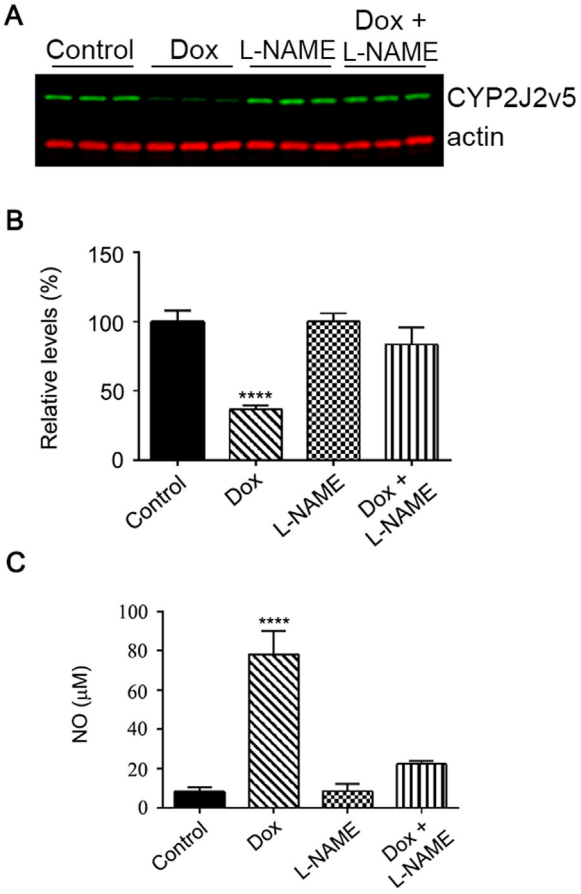

Fig. 4. CYP2J2 down-regulation by endogenously produced nitric oxide. To test the effect of physiological NO on CYP2J2 down-regulation, HeLa cells co-expressing CYP2J2 and doxycycline (Dox) inducible nitric oxide synthase (NOS) were used. Cells were incubated with 10 μM Dox and after 24 h, extracellular media were collected to measure the level of nitrate/nitrite (NOx) before harvesting cells. To inhibit NOS activity, 200 μM L-NAME (LN) was co-incubated with Dox. CYP2J2 levels were measured by Western blotting (A) and the relative expression of CYP2J2 was plotted after normalization using actin (B). NOx levels were determined by Griess reaction (C). $^{****}p < 0.0001$ compared to control group; one-way ANOVA and Tukey's test (n = 3).

Fig. 1B, CYP2J2 activity was measured after short periods of incubation with DPTA. The activity was significantly decreased to 52% even after 20 min of DPTA treatment followed by a slower time-dependent inhibition (Fig. 5A). However, the inhibited activity by DPTA was dramatically restored when the CYP2J2 substrate was added after the media containing DPTA was replaced with fresh media (Fig. 5A). To eliminate the possibility that DPTA may inhibit or quench luminescent signals, DPTA was added during the reaction of metabolized luciferin-2J2/4F12 substrate with luciferin detection reagent and we found no interference of DPTA on product detection.

Alternatively, we examined if stable NO modifications contribute to CYP2J2 degradation. In this experiment, we hypothesized that CYP2J2 protein levels would be continuously decreased after removal of DPTA if stable NO modifications are responsible for the degradation. To test the hypothesis, Huh7 cells were incubated with DPTA for 4 h, washed, and additionally grown for 0, 1, 2, 4, and 6 h more in fresh growth media containing cycloheximide. However, the activity was quickly restored within 1 h similar to Fig. 5A and further protein degradation was not detected (Fig. 5B-D).

3.5. In vitro S-nitrosylation of CYP2J2

Since CYP2J2 activity was quickly restored after removing DPTA-

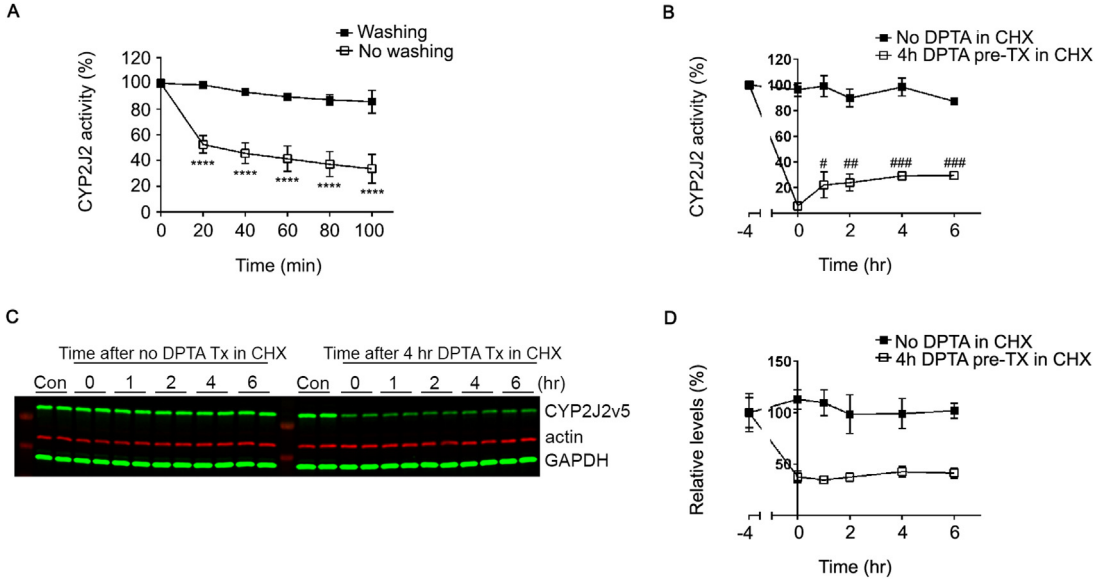

Fig. 5. Reversible NO-medicated modification of CYP2J2. Two groups of Huh7 cells were treated with 500 μM DPTA for 20, 40, 60, 80 and 100 min. After the indicated time points, in one group 30 μM luciferin-2J2/4F12 was added to cell culture media. In the other group, cells were washed with fresh media twice and then the substrate was added. Cells were incubated with the substrate at 37 °C for 20 min and 25 μl of the reaction mixture was transferred to opaque white 96-well plates. Same volumes of Luciferin detection reagent were added and after 20 min, the luminescence was read using the FLUOstar OMEGA microplate reader (A). ****Significantly different from the activity in the washing group at same time point; two-way ANOVA with Sidak's correction ($p < 0.0001$; n = 3). To determine the contribution of stable NO modifications to CYP2J2 degradation, cells were pre-incubated with DPTA for 4 h in the presence of 10 μg/ml CHX. After washing cells, fresh media without DPTA, and containing 10 μg/ml CHX were added and cells were additionally incubated for 1, 2, 4 or 6 more hours. Measurement of CYP2J2 activity (B) and CYP2J2 protein levels by western blotting (C). Fluorescent intensities from panel C were combined with data from two other experiments and plotted as the means ± SD of three independent experiments (D). # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$, compared to the 0 h DPTA-treated sample. Two-way ANOVA and Tukey's test.

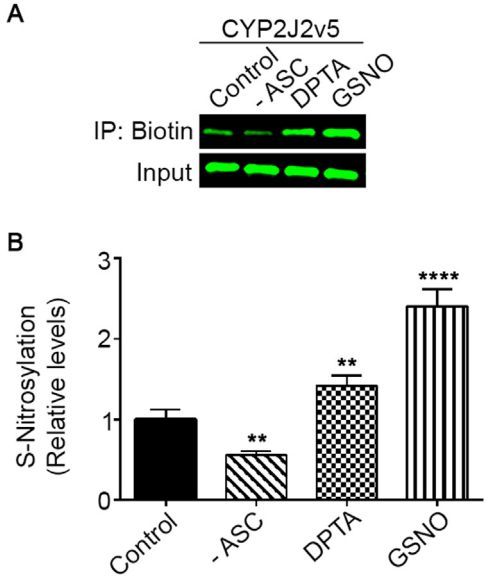

Fig. 6. In vitro nitrosylation of CYP2J2. Microsomes (200 μg) isolated from Huh7 cells expressing CYP2J2v5 were treated with DPTA (100 μM), or GSNO (100 μM) for 30 min at 37 °C. After the treatments, biotin-switch technique was performed as described in Materials and Methods, followed by Western blotting (A). The mean value of each group ± SD was plotted. Significantly different from control (B) (**$p < 0.01$; ****$p < 0.0001$), one-way ANOVA and Dunnett's test (n = 4).

containing media (Fig. 5), we tested whether CYP2J2 enzymes have the capacity to be nitrosylated. For the experiment, microsomes isolated from Huh7 cells expressing CYP2J2v5 were treated with 100 μM of DPTA or GSNO (an S-nitrosylating agent). During labeling of nitrosylated cysteine residues with biotin, ascorbate (ASC) converts S-nitrosothiols to thiols targeted for biotinylation and in this experiment no addition of ASC (-ASC) was used as a negative control. As seen in Fig. 6, DPTA and GSNO treatment showed enhanced levels of nitrosylation of CYP2J2 by 1.4 and 2.5 fold, respectively, compared to the level of the control group.

3.6. Inhibition of NO-mediated CYP2J2 degradation by protease inhibitors

Individual cytochrome P450 proteins have particular half-lives and the enzyme degradation occurs mainly via proteasomal or/and autophagic-lysosomal degradation pathways [32–34]. However, it is not clear whether P450 proteins undergo same degradation pathway in abnormal conditions. To determine the mechanism by which NO-mediated CYP2J2 degradation occurs, Huh7 cells were incubated with lysosomal, proteasomal, or calpain inhibitors in the presence of DPTA.

Inhibition of lysosomal proteolysis via chloroquine had no effect on the down-regulation by DPTA in Huh7 cells (data not shown). However, the proteasome inhibitor bortezomib showed partial but significant restoration of CYP2J2 levels (Fig. 7A, B). Previously, our lab showed that NO degrades CYP2B6 via ubiquitin-dependent proteasomal pathway [14]. By greatly increasing the gain on the IR fluorescence signal, we could see the accumulation of higher molecular mass (HMM) species of CYP2J2 in the presence of bortezomib (Fig. 7D), suggesting ubiquitination of CYP2J2. However, unlike CYP2B6, there were no differences in the level of accumulated higher molecular bands between samples treated with the proteasome inhibitor in the presence or absence of DPTA. To confirm whether ubiquitination is involved in NO-mediated CYP2J2 degradation or not, hemagglutinin-tagged Ubiquitin (HA-Ub) was transiently introduced into Huh7 cells expressing CYP2J2v5. After 24 h posttransfection, cells were incubated for 2 h with DPTA in the presence or absence of bortezomib. Cell lysates were pulled down with V5 antibody-tagged magnetic beads and immunoblotted with the HA-Ub antibody. Consistent with Fig. 7D, no stimulated ubiquitination was found (Fig. 7E). This result suggests that NO-evoked

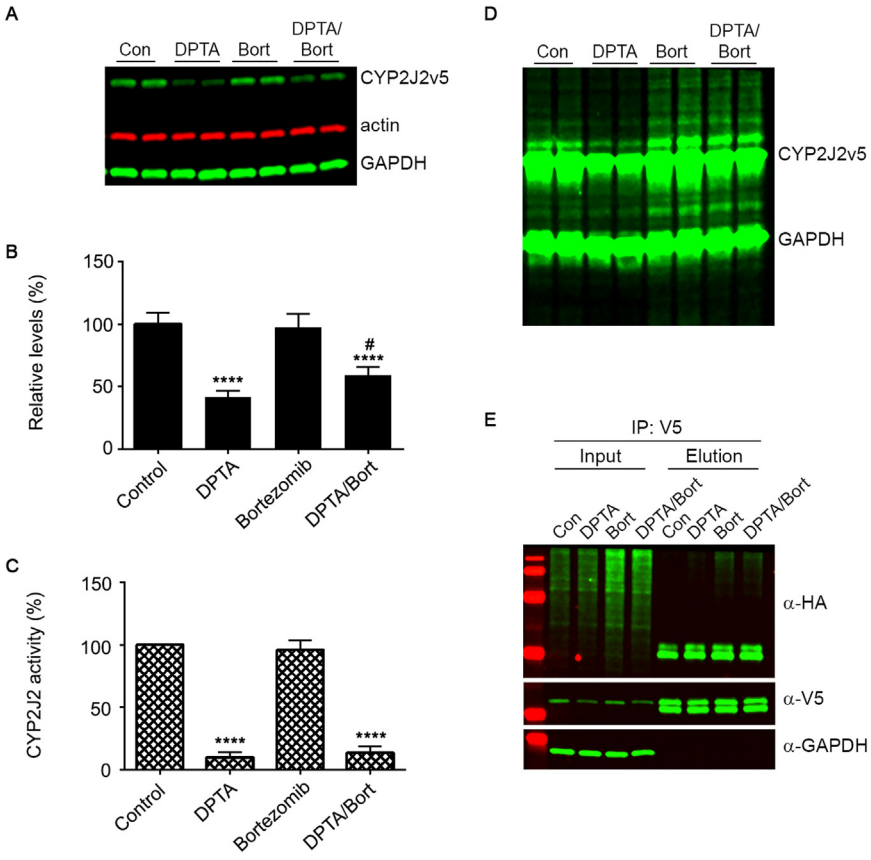

Fig. 7. Effects of bortezomib on NO-mediated CYP2J2 degradation and ubiquitination. Huh7 cells were pretreated with 10 μM bortezomib (Bort), a proteasome inhibitor for 1 h, followed by incubation with or without 500 μM DPTA for 4 h. Cell lysates were subjected to western blotting for CYP2J2 expression (A). After normalization of CYP2J2 protein expression using actin and GAPDH, the mean value of each group ± SD was plotted (B). Cellular CYP2J2 catalytic activity (C). To examine ubiquitination of CYP2J2, higher molecular bands were visualized by increasing the gain on the green IR fluorescence signal (D). After 2 h DPTA treatment, ubiquitination of CYP2J2 was confirmed by immunoprecipitation of CYP2J2v5 in HA-Ub transfected cells followed by immunoblotting against HA-Ub (E). $^{****}p < 0.0001$, compared to control; $\#\ p < 0.05$, compared to DPTA. One-way ANOVA and Tukey's test.

CYP2J2 degradation is ubiquitin-independent although CYP2J2 may undergo ubiquitin-dependent proteasomal degradation in a normal condition.

In addition, the down-regulation of CYP2J2 was also attenuated by calpeptin (Calp), a calpain inhibitor (Fig. 8A, B). However, the inhibitory effect was not detected with calcium chelators inhibiting calpain activity or other calpain inhibitors except for calpain inhibitor III (Cal III) (Suppl. Figs. 2 and 3D). Although Cal III remarkably blocked CYP2J2 degradation by NO, it also inhibited CYP2J2 activity, suggesting Cal III stabilizes CYP2J2 proteins by directly binding to the enzyme (Suppl. Fig. 3D-F). Importantly, the two inhibitors, bortezomib and calpeptin showed no influence on CYP2J2 activity (Figs. 7C, 8C). To determine if the binding of chemicals to P450 inhibits DPTA-mediated degradation, cells were treated with DPTA in the presence of

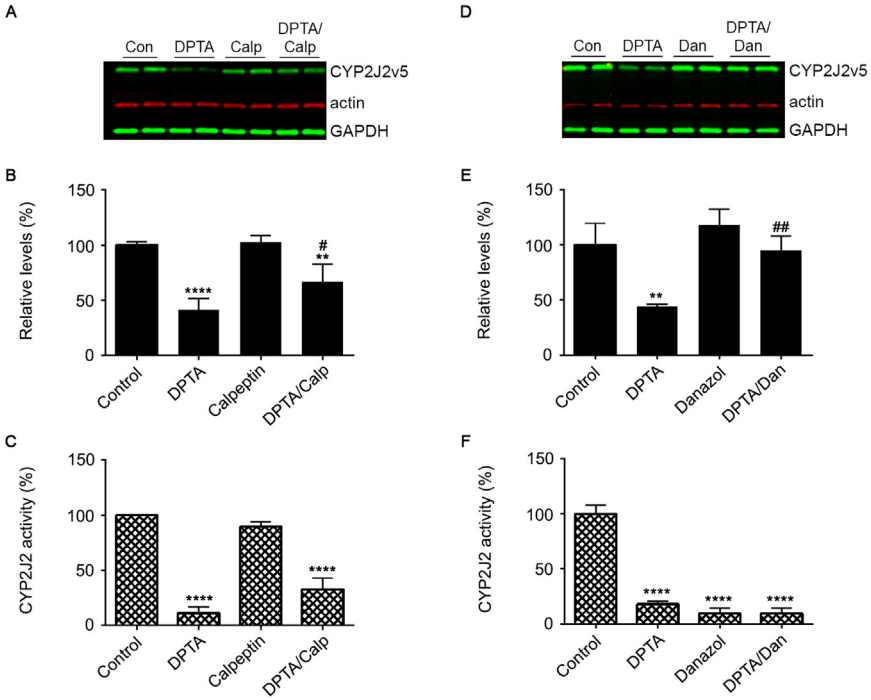

Fig. 8. Inhibition of CYP2J2 degradation by calpain and CYP2J2 selective inhibitors. Huh7 cells were incubated with 500 μM DPTA for 4 h following pretreatment of 50 μM calpeptin (Calp), a calpain inhibitor, or 10 μM danazol (Dan), a potent CYP2J2 inhibitor for 1 h. CYP2J2 levels were determined by Western blotting (A, D) and the mean value of each group ± SD from at least three independent experiments was plotted after normalization using GAPDH and actin (B, E). Activity was measured as described in Materials and Methods. $^{**}p < 0.001$ and $^{****}p < 0.0001$, compared to control; $\#\ p < 0.05$ and $\#\#\ p < 0.01$, compared to DPTA. One-way ANOVA and Tukey's test.

danazol, a potent CYP2J2 inhibitor [26]. With danazol treatment, the activity was remarkably inhibited to less than 10% of the activity of control and DPTA-induced CYP2J2 degradation was almost blocked (Fig. 8D-F).

4. Discussion

Here we demonstrated that nitric oxide (NO) inhibits CYP2J2 activity and also induces down-regulation of the enzyme post-translationally. Although the most well-defined NO mechanism of action involves the activation of soluble guanylate cyclase (sGC) through binding of NO to heme of sGC, posttranslational modification of proteins is also considered as a key NO signaling mechanism resulting in altering protein activity and stability, and subsequent signal transduction [35]. According to several studies, the posttranslational modifications by NO in P450 enzymes include S-nitrosylation on cysteine residues, nitration of tyrosine, and heme nitrosylation [9–11]. While NO has a very short half-life (< 2 s), its oxidative metabolites nitrite (NO_2^-) and nitrate (NO_3^-) have longer half-lives and can be reconverted to NO. In addition, NO can react with superoxide anion to generate peroxynitrite ($ONOO^-$), resulting in nitration of proteins. Due to these inter-convertible characteristics, both nitrosylation and nitration can occur in target proteins by incubating cells with NO donors. In the current study, we used the NO donor DPTA to test NO effects on CYP2J2 regulation. Because of concerns about the stability of DPTA and the rate of NO release, we performed similar experiments in HeLa cells expressing doxycycline-inducible NOS2. As shown in Figs. 1 and 4, CYP2J2 proteins were down-regulated by both DPTA and endogenously produced NO.

Cellular CYP2J2 was rapidly inhibited by application of DPTA, and CYP2J2 activity was quickly restored when DPTA was removed from the media (Fig. 5A). This is likely due to reversible heme nitrosylation, but could also involve nitrosylation on cysteine residues that could modify catalytic activity of CYP2J2. It is generally thought that nitrosylation is reversible and tyrosine nitration is irreversible. However, some proteins stably remained S-nitrosylated even after NO production was inhibited [36]. In addition, it has been reported that nitrated proteins could be rescued from the modification by a denitrase activity [37,38]. In Fig. 5, we tested whether stable posttranslational modification of CYP2J2 by NO could continuously promote the protein degradation even after removing DPTA. However, no further protein degradation was detected when the cells treated with DPTA for 4 h were washed with fresh growth media and incubated additional hours (Fig. 5D). At the same time, CYP2J2 activity was restored by washing to levels commensurate with cellular CYP2J2 protein levels. These data demonstrate that continuous exposure of NO is required for CYP2J2 degradation and suggest that reversible NO modifications such as S-nitrosylation or heme nitrosylation may mediate the degradation and the enzyme activity. However, another possible explanation could be that a stable modification like tyrosine nitration triggers very rapid degradation of modified CYP2J2, such that no continuous degradation could be detected after the removal of DPTA. Although we found that CYP2J2 could be nitrosylated in microsomes isolated from CYP2J2v5-expressing Huh7cells (Fig. 6), we were not able to detect enhanced levels of S-nitrosylation in the cells (data not shown). While this could suggest that nitrosylation does not occur in cells, it could also be due to rapid reversal of the modification in vivo. In addition, it is also possible that NO modification induces other modifications which could alter protein stability. For example, S-nitrosylation of vascular endothelial (VE)-cadherin was required for phosphorylation and its internalization [39]. On the other hand, ERK S-nitrosylation inhibited the activity by preventing its phosphorylation [40]. These studies support that NO modification could induce secondary posttranslational modifications. Therefore, further studies are required to determine types of post-translational modifications and target residues.

To determine the degradation mechanism of CYP2J2, Huh7 cells were treated with DPTA in the presence or absence of protease inhibitors including proteasomal inhibitors bortezomib and MG-132, the lysosomal inhibitor chloroquine, the inhibitor of autophagy 3-methyladenine and the calpain inhibitors calpeptin and calpain inhibitor III. We observed partial but significant inhibitory effects of bortezomib and calpeptin in NO-evoked CYP2J2 degradation (Fig. 7 and Fig. 8). Previously, our lab showed that NO stimulates CYP2B6/2B1 degradation via the ubiquitin-proteasome system [10,14]. Interestingly, however, we were not able to detect stimulated ubiquitination of CYP2J2 in cells treated with DPTA in the presence of bortezomib, compared to cells treated only with bortezomib (Fig. 7D, E). Although ubiquitin conjugation is required for the proteasomal degradation of most target proteins including CYP3A4 [41], a significant subset of proteins undergo ubiquitin-independent proteasomal degradation by the core 20 S proteasome itself without the 19 S regulatory particle (e.g. thymidylate synthase) [42]. In fact, the same proteins can be regulated by both ubiquitin-dependent and –independent pathways depending on the situation [43]. Our data suggest that 1) unlike CYP2B proteins, NO stimulates CYP2J2 degradation via the ubiquitin-independent pathway and 2) NO regulates individual P450 enzymes differently.

Along with bortezomib, MG-132, 3-Methyladenine, and calpain inhibitor III also attenuated NO-mediated CYP2J2 degradation. However, we excluded the data from these last three inhibitors due to their CYP2J2 inhibition properties (Suppl Fig. 3). In many cases, P450 substrates and inhibitors bind to the enzymes and stabilize P450 [44,45]. To determine whether binding of inhibitors to CYP2J2 inhibits the NO-mediated degradation, a potent CYP2J2 inhibitor, danazol was treated together with DPTA. In Fig. 8D-F, while danazol inhibited CYP2J2 activity by 90%, there was no significant differences in protein levels between control (con) and DPTA/danazol groups. These findings suggest that without inhibition of proteases, binding of ligands to CYP2J2 can inhibit the enzyme degradation by NO. Further studies are needed to clarify whether danazol stabilizes CYP2J2 via conformational changes or via inhibition of heme nitrosylation by restricting access of NO to the heme. In addition, these results indicated that extra caution is required in testing function of chemicals that are metabolized by or interact with P450 enzymes. Unlike protease inhibitors that bind to CYP2J2, calpeptin showed no effect on CYP2J2 activity (Fig. 8C). However, no other calpain inhibitors including (2 S,3 S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (EST, also known as E-64d) and PD150606 inhibited CYP2J2 degradation. In addition, although calcium chelators were used to inhibit calpain activity, we were not able to detect similar effects like calpeptin. Giguere (2008) suggested that calpeptin could inhibit proteasome activity in some conditions [46]. Therefore, the effect of calpeptin on CYP2J2 degradation might be caused by its inhibitory activity on proteasome or on unknown proteases.

5. Conclusions

In conclusion, we have demonstrated that CYP2J2 is down-regulated by NO, and that this degradation occurs at least partially via an ubiquitin-independent proteasomal pathway. Because CYP2J2 is involved in the metabolism of both xenobiotics and arachidonic acids (AA), its regulation by NO can cause adverse drug reactions including drug-drug interactions or abnormal AA metabolism. In contrast to P450 regulation by NO, it has been reported that P450 enzymes including CYP1A2 and CYP2J2 regulate NO generation from organic nitrates such as nitroglycerin and isosorbide dinitrate [47]. Although the NO donors are widely used for cardiovascular diseases as vasorelaxant agents, the development of tolerance is a major limitation. Therefore, a deep understanding of the relationship between CYP2J2 and NO will be important for diminishing adverse drug reactions and potentially for long-term use of some NO donor drugs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2018.04.576.

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