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Specific packaging and circulation of cytochromes P450, especially 2E1 isozyme, in human plasma exosomes and their implications in cellular communications

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Abstract

Cytochrome P450 (CYP) enzymes metabolize the majority of xenobiotics and are mainly found in hepatic and some extra-hepatic cells. However, their presence and functional role in exosomes, small extracellular vesicles that are secreted from various cells into extracellular fluids including plasma, is unknown. In this study, we analyzed the expression and biological activity of CYP enzymes in human plasma exosomes. First, we optimized isolation of plasma exosomes and characterized them for their physical properties and quality. The results showed that the purity of exosomes (<200 nm) improved upon prior filtration of plasma using a 0.22 micron filter. We then analyzed the relative level of exosomal CYP mRNAs, proteins, and enzyme activity. The results showed that the relative level of CYP enzymes in exosomes is higher than in plasma, suggesting their specific packaging in exosomes. Of the seven CYP enzymes tested, the mRNA of CYP1B1, CYP2A6, CYP2E1, and CYP3A4 were detectable in exosomes. Interestingly, the relative level of CYP2E1 mRNA was >500-fold higher than the other CYPs. The results from the Western blot showed detectable levels of CYP1A1, CYP1B1, CYP2A6, CYP2E1, and CYP3A4. Our results also demonstrated that exosomal CYP2E1 and CYP3A4 show appreciable activity relative to their respective positive controls (CYP-induced baculosomes). Our results also showed that CYP2E1 is expressed relatively higher in plasma exosomes than hepatic and monocytic cells and exosomes derived from these cells. In conclusion, this is the first evidence of the specific packaging and circulation of CYP enzymes, especially CYP2E1, in human plasma exosomes. The findings have biological and clinical significance in terms of their implications in cellular communications and potential use of plasma exosomal CYPs as biomarkers.

Keywords

Plasma; Exosomes; Cytochrome P450; CYP2E1

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1. Introduction

The first-pass metabolism in the liver is mainly mediated through the family of cytochrome P450 (CYP) isozymes. Of the 57 human CYP enzymes, CYP3A4 contributes to the metabolism of ~45% of pharmaceutical drugs [1]. In addition, CYP2E1 is involved in the metabolism of important xenobiotics such as alcohol, acetaminophen, etc., whereas CYP1A1, CYP1B1, and CYP2A6 are involved in the metabolism of tobacco constituents [1]. These enzymes, especially CYP3A4, are also induced as well as inhibited by several drugs and other xenobiotics [2]. As a result of polydrug prescriptions and the use of alcohol and tobacco products, there is a risk of drug-drug interactions (DDI) [3]. Although the liver is the main site of CYP-mediated drug metabolism and DDI, these enzymes are also expressed in other extra-hepatic cells such as in the gut, kidneys, lungs, monocytes, and brain [4,5]. However, there is no report that CYPs (mRNA or protein) are secreted in extracellular fluids through exosomes and circulated in systemic fluids such as plasma.

Exosomes are small, cell-derived extracellular vesicles (<200 nm), which are secreted from a variety of cells into biological fluids including plasma and cell culture media [6,7,8,9]. Exosomes are gaining importance for prognosis, therapy, and their use as biomarkers for a variety of disease conditions [7,10]. Since the majority of CYP enzymes are expressed in the liver, it is anticipated that the plasma is rich with liver-derived exosomes that contain CYP enzymes. Since CYPs are also found to be expressed in extrahepatic cells, e.g. monocytes, CYP-containing exosomes may also be produced from extrahepatic cells, especially under stress conditions [1,5,11]. Therefore, exosomes circulated in the plasma may come from a variety of cells and may have important biological functions. Moreover, exosomal CYP enzymes can be key players in intercellular communication and modulating drug metabolism in extrahepatic cells since these cells express relatively less CYP enzymes [12]. However, the abundance and the potential physiological role of plasma exosomal CYPs have not been studied yet. In this study, we examined the relative abundance of CYP enzymes in plasma exosomes as well as the relative abundance of CYP2E1 in plasma exosomes, compared with hepatic and monocytic cells and exosomes derived from these cells.

2. Materials and Methods

2.1. Isolation of plasma and cellular exosomes

Plasma was prepared as described earlier [13] from human blood samples of unidentified healthy individuals, which were obtained from Interstate Blood Inc. (Memphis, TN). Exosomes were isolated first by filtering plasma (200 – 500 µl) through a 0.22 micron filter to remove large vesicles (>200 nm) followed by using Plasma Exo Kit (Applied Biosystems, Foster City CA). We also isolated exosomes from two clinically relevant CYP expressing cell lines- hepatic and monocytic cells. The terminally differentiated HepaRG® cells (ThermoFisher Scientific, Grand Island, NY) were maintained in media prepared by the addition of the HepaRG® Tox Medium supplemented with 100 ml of William's Medium E and 1 ml of GlutaMAX. The cells were cultured for 7 days to achieve peak CYP expression. Then the HepaRG maintenance media was replaced with DMEM media supplemented with exosome-free FBS, sodium bicarbonate, non-essential amino acids, and gentamycin, and was further grown for 4 days for optimal secretion of exosomes [14,15]. U937 monocytic

cells were differentiated into macrophages followed by culture in exosome-free FBS in RPMI media for 4 days. Exosomes from these cells were collected from the media using the methods developed earlier [14,16]. Briefly, we collected cell-free exosome-containing fluids, filtered through a 0.22 micron filter, and then used the ExoELISA kit (System Biosciences, Palo Alto, CA). The exosomal pellet was resuspended in water for determination of size and zeta potential of exosomes using Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK) as described earlier.

2.3. Quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR)

The mRNA from the exosomal pellet was isolated using QIAGEN Kit (Valencia, CA) and quantified using Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Fischer Scientific, Rockford, IL). Q-RT-PCR was used to measure the relative mRNA expression of CYP1A1 (Hs01054794_m1), CYP1B1 (Hs00164383_m1), CYP2A6 (Hs00868409_s1), CYP2E1 (Hs00559367_m1), CYP2D6 (Hs00164385_m1), CYP3A4 (Hs00430021_m1), and a cellular house-keeping gene, β -actin (Hs99999903_m1), using the indicated probes from Applied Biosystems. RNA (90 ng) was first reverse transcribed to cDNA using a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA) and then cDNA was amplified in a Step-One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression kit (Applied Biosystems, Foster City, CA). The cycle numbers of each Q-RT-PCR was recorded and analyzed.

2.4. Western blot analysis

The protein level of CYPs in plasma exosomes was determined by loading 5 μ g of protein in 10% SDS polyacrylamide gel followed by western blotting using standard protocol. In brief, the proteins from the gel were transferred to a polyvinylidene fluoride membrane and blocked using Li-Cor blocking buffer (LI-COR Biosciences, Lincoln, NE). The membrane was incubated overnight with primary antibodies (GAPDH Rabbit Mab, Cell Signaling Technology, Danvers, MA; CYP1A1, CYP2E1, and β -actin, Rabbit Mab, Abcam, Cambridge, MA; CYP1B1, CYP2C9, CYP3A4, CPR, and CD63, Mouse Mab. Santa Cruz Biotechnology. Inc. Dallas, TX) at 4°C using appropriate dilution (1:200 – 1:500). After subsequent washing, the blots were incubated with corresponding secondary antibodies (Goat anti-Mouse Mab and goat-anti-Rabbit Mab, LI-COR Biosciences) for 1 hour at room temperature. The blots were scanned with Li-Cor Scanner (LI-COR Biosciences). CD63 (Santa Cruz) was used as an internal loading control for exosomal proteins.

2.5. Measurement of CYP level and enzymatic activity

CYP measurement was performed using absolute spectrum as described previously [17]. Briefly, 5–20 μ l of exosomes were resuspended in 0.1 M Hepes buffer, pH 7.4, and spectra were scanned between 350 and 700 nm. The absorbance at 417 nm was used to determine the CYP concentration. Total protein concentration was determined using a BCA assay. CYP2E1 and CYP3A4 activity assays were carried out as described previously [18,19] by using Vivid® CYP Screening Kit from Life Technologies as recommended by the manufacturer (# P2858, Carlsbad, CA). Briefly, 1–5 μ l of exosomes were incubated with substrate reaction mixtures and the fluorescence was measured using a microplate reader (Cytation™ 5 Cell Imaging Multi-Mode Reader, BioTek, VT). CYP3A4 activity was also

measured using 7-benzyloxyquinoline (7-BQ) as a substrate and H_2O_2 as an oxidant as described previously [20]. This assay was performed using 100 μM 7-BQ and 10 mM H_2O_2 in 0.1 M Hepes buffer, pH 7.4. The fluorescence was measured at λ_{ex} (410 nm) and λ_{em} (510 nm). CYP2E1- and CYP3A4-induced baculosomes were used as a positive control. The specific activity of CYP was determined as the mean fluorescence intensity (MFI)/min/nmol CYP.

2.6. Statistical analysis

Graphs were plotted and two-tailed t-tests were performed wherever appropriate using GraphPad Prism 6 software (GraphPad, San Diego, CA).

3. Results and Discussion

3.1. Physical characterizations of plasma exosomes

First, we improved the purity of plasma exosomes by filtering the plasma using 0.22 micron filters prior to applying the plasma EXOKIT. We used 0.22 micron filters to ensure that we recovered exosomes that are ~ 200 nm in size as defined [6,7,8,9]. The results showed that unfiltered exosomes have a second minor peak and an average size of 119 nm compared to filtered exosomes that have a single peak with an average size of 109 nm (Fig. 1A). Further analysis showed that the filtered exosomes have a significantly higher yield ($92\% \pm 1$) than the unfiltered exosomes ($84 \pm 2\%$) within the range of 200 nm (Fig. 1B). As expected, exosomes from filtered plasma did not show a significant difference in zeta potential compared with unfiltered plasma (-12 ± 1 mV vs. -11 ± 0.9 mV). Overall, these results suggest that the isolation of exosomes by using a prior filtration step improves the yield and quality of exosomes, as well as their characteristics in terms of size, resembling plasma exosomes reported earlier [21].

3.2. Analysis of CYP enzymes in plasma exosomes

We measured CYP concentration using absolute spectra (417 nm) and compared with total protein concentration. Plasma contained 2.2 pmoles CYP in 37.8 μg protein, whereas exosomes contained 0.22 pmoles CYP in 1.71 μg protein (Fig. 2). The analysis for their relative abundance suggests that 4.5% of the plasma proteins are in exosomes, whereas 10% of the plasma CYPs are in exosomes.

We performed Q-RT-PCR to detect whether individual CYP mRNA are packaged in plasma exosomes from two donors. Of the seven CYPs (1A1, 1B1, 2A6, 2C9, 2D6, 2E1, and 3A4) tested, only CYP1B1, CYP2A6, CYP2E1, and CYP3A4 showed detectable levels (Fig. 2), except CYP3A4 in donor 2. The remaining CYPs as well as the house-keeping genes GAPDH and β -actin were undetectable in plasma exosomes. Using the same amount of mRNA, CYP2E1 was amplified at ~ 30 cycles, while CYP1B1, CYP2A6, and CYP3A4 was amplified at >40 cycles. Considering the similar RT-PCR efficiency for these CYPs, the result suggests that the relative abundance of CYP2E1 is >500 -fold higher than in the other CYPs. Furthermore, we carried out western blotting of exosomal CYPs, cytochrome P450 reductase (CPR), GAPDH, β -actin, and exosomal marker protein CD63 from two donors. Interestingly, while CYP1B1, CYP2A6, CYP2E1, CYP3A4, and CD63 showed detectable

bands (Fig. 2), CYP1A1, CYP2C9, CYP2D6, CPR, GAPDH, and β -actin did not show detectable bands (data not shown). In summary, a relatively high P450 concentration in exosomes and the presence of mRNA and proteins of CYP enzymes clearly suggest a selective packaging of specific CYP enzymes, especially CYP2E1.

3.3. Functional activity of exosomal CYP2E1 and CYP3A4

Next, we sought to determine whether exosomal CYP enzymes are metabolically active. We used Vivid® assays to determine the activity of two representative enzymes CYP2E1 and CYP3A4. CYP2E1 showed a linear activity with time, while CYP3A4 did not show detectable activity using this assay (Fig. 3A). Since the protein level of CPR was undetectable, we used H₂O₂-dependent CYP3A4 assay using 7-BQ as substrate, which is known to show CYP3A4 activity, albeit several fold lower than NADPH-dependent activity [20]. Indeed, CYP3A4 showed detectable activity with H₂O₂ (Fig. 3B). Further analysis showed that CYP2E1 has specific activity of 6.16 MFI/min/pmol CYP, which is approximately 50-fold lower than that of the positive control, CYP2E1-induced baculosomes. Similarly, CYP3A4 showed a specific activity of 28.2 MFI/min/pmol CYP, which is approximately 8-fold lower than that of CYP3A4-induced baculosomes. Compared to the control, very low NADPH-dependent CYP2E1 activity could be due to extremely low (undetectable) level of CPR in the exosomes. It can be noted that CYP2E1 does not show activity using H₂O₂. On the other hand, H₂O₂-dependent CYP3A4 activity was only 8-fold lower than the NADPH-dependent activity, which is consistent with the reported finding [20].

3.4. Comparison of plasma exosomal CYP2E1 with cellular and cell-derived exosomal CYP2E1

Further, the relative cellular and exosomal CYP2E1 mRNA and protein levels from HepaRG and U937 monocytic cells were analyzed (Fig. 4A–B). Using the same amount of mRNA (20 ng), the hepatic cellular CYP2E1 was amplified at ~30 cycles, while exosomal CYP2E1 mRNA was amplified at ~34 cycles (Fig. 4A). Interestingly, both cellular and exosomal CYP2E1 mRNAs from U937 cells were amplified at ~34 cycles. It is intriguing that the plasma exosomal CYP2E1 mRNA was also amplified at ~30 cycles (Fig. 2). These results suggest that CYP2E1 mRNA is highly abundant in plasma exosomes, perhaps as much as in hepatic cells. Upon comparison with the cycle numbers, considering similar RTPCR efficiency, results suggest that HepaRG® cells express >10-fold higher CYP2E1 mRNA than HepaRG-derived exosomes, while U937 cell-derived exosomes express similar to the cellular level of CYP2E1 mRNA. It can be noted that the total cellular mRNA level is generally much higher than the total exosomal mRNA level. Similarly, Western blot (5 μ g each) showed a much higher level of plasma exosomal CYP2E1 protein than cellular as well as exosomal CYP2E1 protein levels from both HepaRG® and U937 cells. As expected, the levels of exosomal CYP2E1 derived from HepaRG® and U937 cells were lower than the respective cellular CYP2E1, at least suggesting the packaging of CYP2E1 in exosomes from these cells. It can be noted that both HepaRG and U937 cells contain a ~25-fold higher level of total proteins than the exosomes isolated from the media that were used to grow these cells.

3.5. Biological significance of exosomal CYP enzymes

The formation of exosomes in cells is highly regulated, and exosomes are known to package specific proteins, mRNA, and microRNA to deliver at different sites through the systemic circulation of biological fluids such as plasma [6,22]. The presence of relatively high levels of specific CYPs, such as CYP2E1, CYP1B1, CYP2A6, and CYP3A4 suggests a specific role of these enzymes at distant sites that may be translocated through the circulation in plasma.

CYP2E1 is mainly expressed in the liver cells and metabolizes alcohol and acetaminophen, causing liver toxicity upon binge drinking and acetaminophen overdose [23]. Thus, CYP2E1 in the plasma exosomes appears to be derived from hepatic cells. This assumption is based on the finding that ethanol induces the release of exosomes from hepatocytes as a result of ethanol metabolism by CYP2E1 leading to oxidative stress [24]. It is evident from our study that both hepatic, and to a lesser extent, monocytic cell-derived exosomes, contain CYP2E1 mRNA and protein. Our previous study has shown the presence of CYP2E1 in monocytic cells, which is further induced by alcohol [11,25]. In addition, intriguing findings include a several hundred fold (10 cycles) higher CYP2E1 mRNA expression than the other CYPs in plasma exosomes, as well as a relatively higher expression of CYP2E1 in plasma exosomes than in monocytic cells [26]. This finding suggests that the packaging of CYP2E1 mRNA into plasma exosomes is highly regulated. The results also suggest that CYP2E1-containing exosomes are secreted from hepatic as well as other extrahepatic cells, and accumulate in circulating plasma.

CYP2E1-containing exosomes are likely to be translocated to other organs/cells for specific purposes that are yet to be discovered. It is likely that plasma exosomes are circulated throughout the body, including the brain, and deliver metabolically active CYP2E1 that can metabolize xenobiotics such as alcohol and pharmaceutical drugs, as well as endogenous compounds. The metabolism of these compounds by exosomal CYP2E1 may cause the production of reactive oxygen species-mediated toxicity to the host cells. The argument that plasma exosomal CYP2E1 is delivered to other cells where it metabolizes alcohol and other xenobiotics leading to cellular toxicity, could explain known alcohol-induced cellular injury of extrahepatic cells [27]. In addition to its biological significance, plasma exosomal CYP2E1 may also have clinical significance. Plasma exosomes enriched with CYP2E1 can be used as biological markers under specific conditions such as alcohol use, acetaminophen toxicity, and damage to the liver and other tissue/organs. It is widely known that CYP2E1 is induced in the liver in chronic alcohol users as well as in acetaminophen overdose, which ultimately causes liver damage [28,29]. Our previous study has also shown that alcohol-induced CYP2E1 causes oxidative stress-mediated toxicity in blood (monocytes) and brain (astrocytes) cells [30].

CYP1B1 and CYP2A6 are mainly expressed in lung cells and to some extent in the liver and other extra-hepatic cells such as blood macrophages and brain cells[1,5]. Thus, the source of plasma exosomal CYP1B1 and CYP2A6 appears to be lung and/or liver cells. They metabolize tobacco constituents—benzo(a)pyrene and nicotine, respectively—and are responsible for smoking-mediated carcinogenesis as well as liver toxicity [31,32]. In addition to cancers, CYP1B1 is known to be induced under many disease conditions and has

been implicated as an important biological marker for the predictive diagnosis of developmental disorders [33]. Plasma exosomes containing CYP2A6 may be used as biological markers for nicotine dependence, because it is known to be induced by nicotine and rapidly metabolizes nicotine in the liver [33]. Among the important drug-metabolic CYP enzymes, CYP3A4 showed detectable levels of both mRNA and proteins, as well as H₂O₂-dependent enzyme activity. This finding has clinical significance with respect to drug metabolism and DDI, because it is the major drug-metabolic CYP enzyme [2,3]. Drug regimens are formulated based upon drug metabolism in the liver; however, drug metabolism by plasma exosomes is not accounted for during drug development and may cause drug toxicity and DDI. Plasma CYP3A4 may also be transported to other organs/tissues and may metabolize drugs in the targeted diseased organ/tissue, which may lead to reduced drug efficacy and increased drug metabolite-mediated toxicity. Plasma exosomal CYP3A4 is also likely to be used as a biological marker under specific conditions such as liver diseases and the use of pharmacological and illicit drugs. CYP3A4 is induced by several therapeutic drugs such as phenobarbital, rifampin, ritonavir and herbal products, namely St. John's Wort, and drugs of abuse such as alcohol [34,35].

In conclusion, this study presents the first evidence of the presence of functional CYP enzymes in exosomes. More importantly, the relatively high abundance of CYP1B1, CYP2A6, CYP2E1, and CYP3A4, suggests that their important role in the metabolism of alcohol, tobacco, and other drugs is not limited to their primary site, the liver. Moreover, our data predict that the level of CYP2E1 in plasma exosomes is very high and is sufficient to cause a physiological effect when fused with naïve cells. This finding has biological and clinical significance in terms of examining the role of plasma exosomal CYPs in extrahepatic cells and their use as biological markers under disease and other conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Exosomes were isolated and characterized from human plasma as well as from hepatic and monocytic cells.
- Detectable levels of CYP1A1, CYP1B1, CYP2A6, CYP2E1, and CYP3A4 are present in human plasma exosomes.
- The relative level of CYP2E1 is much higher than the other CYP enzymes.
- Plasma exosomes contain higher level of CYP2E1 than the exosomes derived from hepatic and monocytic cells.
- The circulating exosomal CYPs, especially CYP2E1, in plasma appear to have physiological role in extrahepatic cells.

Fig. 1. Physical characteristics of plasma exosomes using Zetasizer

A. Representative plots from Zetasizer showing the distribution of exosomes at different sizes with and without filtration using 0.22 micron filter. **B.** Analysis of exosomal size (30–100 nm and <200 nm) with and without filtration. Average size and zeta potential were determined from three different samples.

Fig. 2. Analysis of CYP enzymes using absolute spectra, Q-RTPCR, and Western blotting

Absolute spectra was obtained between 350 and 700 nm and absorbance at 417 nm (low-spin CYP heme peak) was used to determine the concentration of CYP. The CYP (in nmol/ml) and protein (mg/ml) quantifications presented are representative of at least three different determinations. Q-RTPCR was performed to determine the relative mRNA level of CYP enzymes from two donors. The relative cycle numbers (mean $\pm$ SEM) is presented from three independent determinations. Western blot of CYP enzymes from two donors are presented.

Fig. 3. Biological activity of exosomal CYP2E1 and CYP3A4

(A) CYP2E1 was assayed using standard Vivid assay (NADPH-dependent activity) and (B) CYP3A4 was assayed using H_2O_2 -dependent debenzoylation of 7-BQ. Exosomes (1–5 μ l) and baculosomes (1–2 μ l) were used for this assay. Representative graphs from at least three determinations for activity (time course) are presented. Specific activity from exosomes and baculosomes were determined and presented.

Fig. 4. Comparison of plasma exosomal CYP2E1 with CYP2E1 from hepatic and monocytic cells and exosomes derived from these cells

Relative cycle numbers of mRNA (using 20 ng) (A) and protein level (using 5 μ g) (B) of CYP2E1 are shown from plasma exosomes, HepaRG and U937 cells, and exosomes isolated from these cells. β -actin and CD63 are marker proteins from cells and exosomes, respectively. The relative cycle numbers (mean $\pm$ SEM) is presented from three independent determinations.