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Short communication

Characterization of release profile of ornithine carbamoyltransferase from primary rat hepatocytes treated with hepatotoxic drugs: Implications for its unique potential as a drug-induced liver injury biomarker

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ABSTRACT

Ornithine carbamoyltransferase (OCT) is a mitochondrial protein expressed primarily in the liver. It has been shown that, like alanine aminotransferase (ALT), OCT is released from damaged hepatocytes in rats and humans, which has given rise to the possibility that OCT might provide a diagnostic biomarker of various forms of liver damage, including drug-induced liver injury (DILI). However, OCT release characteristics in DILI, as well as their diagnostic advantages, remain elusive. Therefore, this study aimed at clarifying whether and how OCT is released from rat primary hepatocytes *in vitro* using seven potentially hepatotoxic drugs. The results showed that OCT releases from damaged hepatocytes were observed for all tested drugs, and that those releases were not associated with mitochondrial membrane proteins. It should be underscored that the release dynamics were significantly larger than those of ALT. Furthermore, unlike ALT, the maximum OCT release levels showed differences depending on the drug being tested, suggesting that OCT release was susceptible to toxicity mechanisms. Taken together, these unique release characteristics highlight the possibility that OCT could provide a promising DILI biomarker that might contribute not only to diagnostic accuracy improvements, but also to a better understanding of toxicity types in clinical and drug development settings.

1. Introduction

Drug-induced liver injury (DILI) refers to a serious adverse drug reaction, and is thus a major cause of drug development failure or drug withdrawals from the market [1,2]. As part of efforts to detect such adverse reactions, it has long been acknowledged that alanine aminotransferase (ALT) and aspartate transaminase (AST) serve as well-established liver injury biomarkers. Although both are released from damaged hepatocytes, ALT is more often considered a primary indicator of hepatocyte injury due to the fact that its expression profile is more liver-restricted than that of AST.

However, several problems related to DILI diagnosis utilizing these classical biomarkers remain unresolved. One is a potential risk for false positive results. For example, it has been reported that benign serum ALT elevation sometimes accompanies heparin or cholestyramine treatments [3,4], and that serum ALT elevation is often observed in hypothyroidism [5]. Another is that the ALT level is *per se* incapable of providing any insights into the mechanisms causing DILI. Therefore, it is necessary to develop new DILI biomarkers, not only for diagnostic accuracy improvement, but also for making possible less-invasive pathological condition evaluation.

One promising blood-based biomarker candidate for liver injury examination is ornithine carbamoyltransferase (OCT). OCT is a mitochondria matrix urea cycle enzyme that is primarily expressed in hepatocyte. It has been shown that, as with ALT, OCT is released into the blood in various rat liver dysfunction models [6,7], and that blood OCT elevation has been also observed in patients harboring liver diseases [8]. Moreover, in the rat models, OCT release dynamics are apparently larger than those of ALT or glutamate dehydrogenase [6,7]. Based on these findings, OCT can be expected to have significant potential for use as a sensitive DILI blood biomarker.

In light of the above, and while noting that OCT release profiles in various DILI types have yet to be investigated, the present study sought to clarify whether and how OCT is released from rat hepatocytes during drug-induced cytotoxicity *in vitro* using several drugs that have been known to cause DILI in clinical settings.

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2. Materials and methods

2.1. Drug exposure to primary rat hepatocytes

Amiodarone (AMIOD) (Sigma, St. Louis, MO, USA), amlodipine (AMLDP) (Wako, Osaka, Japan), celecoxib (CEL) (Sigma), fluoxetine (FLX) (Wako), imipramine (IMP) (Sigma), Tamoxifen (TAM) (Wako), and troglitazone (TRG) (Wako) were used in this study based on the criteria shown in the supplemental information. Rosiglitazone (Wako) and raloxifene (Tokyo Kasei, Tokyo), which have been considered as DILI-negative agents, were also used for comparison. IMP was dissolved in sterile water, and others were dissolved in dimethylsulfoxide (DMSO).

The Animal Research Committee of Chiba University approved the study protocol. Primary rat hepatocytes were prepared from Sprague-Dawley male rats (5–7 weeks old, Japan SLC, Shizuoka, Japan) based essentially on a collagenase two-step digestion method, after which they were re-suspended in Medium K (Table S1). The cells were seeded in a 96-well plate, 4 h after which the medium was replaced with Medium O (Table S1). Twenty-four hours after cell seeding, the medium was changed to Medium S containing either DMSO (0.5%) or one of test drugs (which was set at time = 0).

To examine time-dependent OCT release profiles, the final concentrations of each drug were set at 250 μM. Small samples of each culture medium were obtained at time 0, 0.5, 1, 2, 3, 4, 6, 8, and 12 h. In concentration-dependent OCT release analyses, the concentration range of each drug (see figure legend) was determined based on the results of preliminary experiments. The final DMSO concentration of AMIOD treatment was 2% due to its solubility limitation.

Culture medium samples were collected at the peak time for each drug, and then used for enzyme detection experiments.

2.2. Determination of cytotoxicity level

The ALT release level in culture medium was determined using a Transaminase CII-test kit (Wako) according to the manufacturer's protocol. Similarly, the OCT release level in a culture medium sample was determined using an enzyme-linked immunosorbent assay (ELISA), developed by YAMASA Corporation (Chiba, Japan) [6,7]. These release levels were expressed in terms of fold changes (which are defined as Rf_{ALT} and Rf_{OCT}, respectively) relative to those observed in a DMSO-treated cell culture medium at the corresponding time point.

The maximum ALT or OCT release level (which are referred to as $Rf_{max,ALT}$ or $Rf_{max,OCT}$, respectively) and their half maximal (50%) release concentrations (which are referred to as $RC_{50,ALT}$ or $RC_{50,OCT}$, respectively) were tentatively estimated using the following equation:

$$Rf(c) = \mathrm{Rf}_{\min} + \frac{\mathrm{Rf}_{max} - \mathrm{Rf}_{min}}{1 + \left(\frac{c}{\mathrm{RC}_{50}}\right)^{n}},$$

where "c" indicates drug concentration used, "$\mathrm{Rf}_{\min}$" indicates the estimated minimum release fold for each drug treatment (=1), and "n" indicates Hill coefficients. The calculation was performed using DeltaGraph software (Nihon Poladigital, Tokyo, Japan).

2.3. Western blotting analysis

The presence and levels of the OCT, the carbamoyl phosphate synthetase 1 (CPS1), the cytochrome c oxidase subunit IV (COX IV), the translocase of outer mitochondrial membrane 20 (Tom20), and the albumin protein in cell homogenates or culture medium samples were examined by Western blotting. Antibody information was summarized in Table S1.

2.4. Others

Detailed information of materials and methods are provided in the Supplemental materials.

3. Results and discussion

To determine whether OCT was actually released in drug-induced hepatocyte toxicity, and if so, whether it was preceded or followed by ALT release, time-dependent release profiles were examined for each drug. The results showed that OCT release was clearly detected in all drug exposures except for rosiglitazone and raloxifene (Fig. S1), and that the releases were observed as early as 30 min after the drug exposure with the following peak release time points: 1 h in CEL treatment, 6 h in AMIOD or IMP treatment, and 2 h in others (Fig. S2). These time-course profiles of OCT release were found to be quite similar to those of ALT.

Next, in order to clarify OCT release characteristics, concentration-dependent OCT releases from rat hepatocytes treated with the test drugs at each peak release time point were examined and compared with those of ALT. For quantitative analysis, the RC_{50} and Rf_{max} values of OCT and ALT releases were tentatively estimated. The results showed that, based on comparison of the estimated $RC_{50,OCT}$ and $RC_{50,ALT}$ values, the minimum drug concentrations for OCT release were comparable to those of ALT (Table 1).

On the other hand, Rf_{OCT} was significantly higher than Rf_{ALT} in all drug treatments (Fig. 1). Furthermore, while the $Rf_{max,ALT}$ values did not vary significantly among the drugs tested (9- to 13-fold), the $Rf_{max,OCT}$ values showed larger difference depending on the drug (24- to 79-fold) (Table 1). Accordingly, the IMP Rf_{max} ratio ($Rf_{max,OCT}$/$Rf_{max,ALT}$) and the AMIOD $Rf_{max,OCT}$/$Rf_{max,ALT}$ were significantly lower than others and those of CEL, FLX, and TAM, respectively.

Taken together, these results suggest that while OCT release time profiles and their estimated minimum responsive doses are similar to those of ALT in drug-induced cytotoxicity, the relative OCT release levels apparently show a greater dynamic range when compared with those of ALT release. Because these larger OCT release dynamics are likely to provide clear signal-to-noise ratios in clinical liver tests, it is possible that serum OCT monitoring, in conjunction with the serum ALT, will allow clinicians to definitely diagnose hepatic damage at an earlier time point and with enhanced accuracy.

Furthermore, it should be underscored that, in contrast to relatively homologous ALT release profiles among the seven drug treatments, OCT release creates specific drug-dependent profiles. Therefore, it can be assumed that extensive OCT release may be associated with specific molecular processes, which then brings to mind the possibility that OCT release profiles could provide indicators of certain type(s) of hepatotoxicity. While this idea might be considered somewhat speculative at present, if demonstrated, OCT/ALT release ratio calculations can be expected to provide an objective basis for the classification of liver injury types.

To gain mechanistic insight into how OCT is released from injured rat hepatocytes, the release of OCT and other mitochondrial proteins were examined simultaneously by Western blotting (Fig. 2). The results showed that, as expected, OCT was released from hepatocytes treated with AMIOD, TAM, CEL or IMP, but not with DMSO. Additionally, it was found that CPS1, which is another mitochondrial matrix protein, exhibited a behavior parallel to that of OCT. In contrast, even under the same experimental conditions,