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Arginase release by primary hepatocytes and liver slices results in rapid conversion of arginine to urea in cell culture media

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Abstract

Precision-cut liver slices and primary hepatocytes constitute suitable model systems for studying liver function. Frequently, urea cycle activity is used as a parameter to determine hepatocyte viability. Liver cells contain high levels of the urea cycle enzyme arginase, which converts arginine into urea and ornithine. Arginase can leak from the cells into the supernatants, converting arginine directly to urea and in this way circumventing the urea cycle. In this study, a hepatocellular cell line (HepG2 cells), a primary rat hepatocyte culture, and precision-cut rat liver slices were compared with respect to arginase leakage in the media by determining arginine conversion into urea. HepG2 cells did not show arginine conversion to urea during 24 h incubations. In contrast, in both precision-cut liver slices and primary rat hepatocytes all arginine was converted to urea. Arginase activity was confirmed by showing that freshly added arginine to the cell-free supernatants again was converted to urea. In conclusion, when choosing urea production of primary hepatocytes cultures as a viability indicator, one has to take into account that arginase can leak from the cells into the supernatant. This can lead to an overestimation of the viability of the cells, since arginase converts arginine into urea without involvement of the urea cycle. We suggest using an extra incubation in an arginine-free buffer supplemented with ornithine and NH_4Cl. In addition, arginase leakage can lead to depletion of the supernatant of arginine in primary hepatocytes cell cultures. This might have implications for studying cellular activities where arginine is involved, like, e.g. nitric oxide (NO) production.
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Keywords: Rats; Hepatocytes; Arginase; Urea; Precision-cut liver slices

1. Introduction

Isolated and cultured primary hepatocyte cell systems constitute suitable model systems for studying liver functions such as acute phase protein synthesis and phase I and II detoxification reactions. Frequently, urea synthesis is used as a viability indicator for primary hepatocyte cell cultures (Dabos et al., 2004; Henkens et al., 2007; Muller et al., 2004; Sugimachi et al., 2004; van Poll et al., 2006) and precision-cut liver slice cultures (Day et al., 1999; de Kanter et al., 1998; Khong et al., 2007; Maas et al., 2000; Olinga et al., 2005; t Hart et al., 2005). Urea synthesis from primary hepatocytes seems to be a valid viability indicator, since mitochondrial transmembrane transport of the urea cycle intermediates ornithine and citrulline is involved in the urea cycle (Nissim et al., 2005; Satrustegui et al., 2007; Hirs and Rittenberg, 1950). Accordingly, the rate of appearance of urea is correlated with urea cycle activity and, hence, the viability of the cell system.

So far, two methods have been used to determine urea synthesis as a viability indicator in primary hepatocyte cultures. One involves the production of urea by hepatocytes in an arginine-free ornithine-containing buffer. Urea

Abbreviations: DMEM, Dulbecco's modified Eagle's media; ELISA, enzyme-linked immuno sorbent assay; FCS, fetal calf serum; HRP, horse radish peroxidase; NO, nitric oxide; PBS, phosphate buffered saline.

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production is measured and correlated with cell viability (Day et al., 1999; Khong et al., 2007). The other one involves the determination of urea concentrations in the supernatants of arginine-containing media of cell cultures (de Kanter et al., 1998; Muller et al., 2004; Sugimachi et al., 2004).

One of the enzymes involved in the urea cycle is arginase (L-arginine ureohydrolase EC 3.5.3.1), which hydrolyses arginine to ornithine and urea. Rat liver contains a potent arginase in the cytoplasm of the periportal hepatocytes, which is known as the A1 isoenzyme of arginase, also known as 'liver type' (Herzfeld and Raper, 1976). Since hepatocytes contain high levels of arginase, it is possible that arginase is released from the cytoplasm of dying cells. If present in the supernatants of the hepatocytes it might deplete the media of arginine by its hydrolysation into urea. This will result in high levels of urea and an overestimation of urea cycle activity. This study shows the presence of arginase in cell culture media of primary hepatocyte cultures and precision-cut liver slice cultures.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM, containing 0.398 mM arginine), RPMI-1640 (containing 1.15 mM arginine), fetal calf serum (FCS), streptomycin/penicillin and glutamine were purchased from Life Technologies, Breda, The Netherlands.

2.2. HepG2 cell culture

Human hepatoma HepG2 cells (ATCC HB-8065) were maintained in DMEM supplemented with 10% heat-inactivated FCS with 2 mM glutamine and streptomycin/penicillin in Costar T75 flasks (Corning-Costar, Schiphol-Rijk, The Netherlands) at 37 °C in a humidified atmosphere of 95% air-5% CO_2. Before each experiment, HepG2 cells were incubated during 24 h in RPMI-1640 media with 10% heat-inactivated FCS. All experiments were carried out in six wells plates.

2.3. Primary hepatocyte culture

Adult male outbreed Sprague–Dawley rats (about 200 g; food and water *ad lib*) (Iffa Credo Brussels, Belgium) were anaesthetised by *i.p.* injection of sodium pentobarbital solution (0.1 ml/100 g body weight). Intact hepatocytes from adult male outbreed Sprague–Dawley rats were isolated by collagenase perfusion according to De Smet et al. (1998). Hepatocytes (1.6×10^6 viable hepatocytes; viability was checked by the trypan blue exclusion assay) were seeded in 5 ml DMEM containing 10% heat-inactivated FCS (v/v) and penicillin/streptomycin on 6 cm Petri dishes. The cells were allowed to attach to the Petri dishes during 4 h at 37 °C in an atmosphere of 95% air and 5% CO_2 at a relative humidity of 100%. Subsequently, the cultures were incubated during 24 h in the same atmosphere and media. Albumin secretion was used as a viability indicator.

2.4. Precision-cut liver slice culture

Precision-cut liver slices (thickness 100 μm) were prepared from adult male Wistar rat liver. Rats were anaesthetized by *i.p.* injection of sodium pentobarbital solution (0.1 ml/100 g body weight). Before extraction, the liver was perfused with ice-cold PBS. Subsequently, cores with a diameter of 6 mm were prepared and inserted in the tissue slicer (Brendal Vitron Tissue Slicer), containing RPMI-1640 with penicillin/streptomycin. All media were oxygenated with carbogen. During the incubations, the media contained 10% heat-inactivated FCS. After a pre-incubation of 2 h in 2 ml oxygenated media the media were replaced with 2 ml fresh media and incubated for 24 h. Tissue morphology was checked by light microscopy and cell viability was determined by albumin secretion using ELISA.

2.5. Arginase activity assay

Directly after the incubations the supernatants were divided in two portions. One portion was immediately frozen and stored at −80 °C for urea determination. The second portion was collected in 1.5 ml Eppendorf tubes and centrifuged at 13,000 rpm during 15 min. Subsequently, the media were separated from the pellets and frozen at −80 °C. For the arginase activity assay, the supernatants were thawed and arginine was added (1.4 mM to the RPMI-1640 supernatants and 0.4 mM to the DMEM supernatants). The media were incubated during 8 h at 37 °C followed by urea and arginine determinations.

2.6. Urea assay

The urea concentrations in the media were determined colorimetrically after reaction with diacetyl monoxime, using a kit provided by Sigma Procedure No. 535 (Zwijndrecht, The Netherlands).

2.7. Arginine determination by HPLC

The arginine concentrations in the media were determined with HPLC, using *ortho*-phthaldialdehyde as derivatisation reagent and L-norvaline as internal standard (both from Sigma Aldrich). The method was adapted from van Eijk et al. (1993). The detection level for arginine was 2 pmol/L.

2.8. Albumin ELISAs

Albumin levels in cell culture supernatants were determined by enzyme-linked immuno sorbent assay (ELISA). Sheep anti-rat albumin-IgG, HRP-labelled anti-rat

albumin-IgG and rat albumin antigen were from Biogenesis Ltd. (Pool, England). Rabbit anti-human albumin and HRP-labelled rabbit anti-human albumin were from Dade Behring, Leusden, The Netherlands. Human albumin was obtained from Sigma Chemical Co., Aalsmeer, The Netherlands. A 96-well flat bottom EIA/RIA plate (Corning Life Sciences B.V., Schiphol-Rijk, The Netherlands) was coated overnight with primary antibody diluted in phosphate-buffered saline (PBS). Plates were washed after every step with 0.1% (v/v) Tween-20 (Merck Eurolab B.V., Roden, The Netherlands) in 0.1 M PBS, pH 7.4. Subsequently, the plates were blocked with 5% (w/v) Protifar (Nutricia, Zoetermeer, The Netherlands) in 0.1 M PBS, pH 7.4 during 90 min. Then samples and antigen were incubated in 0.1% (v/v) Tween-20 in 0.1 M PBS, pH 7.4 during 90 min. Subsequently, the plates were incubated with HRP antibodies in 0.1% (v/v) Tween-20 in 0.1 M PBS, pH 7.4 during 90 min. Finally, a colorimetric reaction was carried out by addition of 100 μl undiluted 1-Step Ultra TMB-ELISA (Pierce, Rockland IL, USA). The reaction was stopped with 50 μl of 2 M sulphuric acid and the absorbances were measured at $\lambda = 450$ nm.

2.9. Statistical evaluation

Statistical analysis was performed by using multiple pair-wise comparisons between groups using the Student's *t*-test. *P*-values below 0.05 were considered statistically significant.

3. Results

Cell culture supernatants ($n = 6$) were analysed for both arginine and urea concentrations. No significant urea synthesis was observed in the HepG2 cell cultures. Approximately 2.3% of arginine which was present in the media (original concentration 0.40 mM in DMEM), was consumed during the incubation.

In the media of the precision-cut liver slice incubations and the incubations of primary hepatocytes, arginine concentrations were below detection limit of the HPLC method (<2 pM). The initial concentration of arginine was 1.15 mM in RPMI-1640 for precision-cut liver slices and 0.40 mM in DMEM for primary hepatocytes. Different media were used for precision-cut liver slice culture, because cell cultures were optimised in different media.

When the reduction of arginine levels in both supernatants of the primary hepatocytes culture and the precision-cut liver slice culture were compared to the urea concentrations of the media it was found that arginine was completely converted into urea (Fig. 1 and Table 1). To confirm the presence of arginase activity, arginine was added to the arginine-depleted supernatants to restore the initial concentration of arginine of the media. After 8 h incubations at 37 °C, arginine and urea concentrations were determined. In the supernatants of precision-cut liver slices, the arginine concentrations were decreased to below detection limits. All arginine was again converted to urea (Table 2). In addition, 79% of arginine in the primary hepatocyte supernatants was converted to urea (Table 2).

Cell viability was determined by liver-specific cell morphology by light microscopy for the precision-cut liver slices and the trypan blue exclusion test, followed by cell counting of viable cells for the primary hepatocytes. More than 99% of the hepatocytes were viable according to the trypan blue test. In addition, all cultures were tested for albumin secretion. In the primary rat hepatocyte culture albumin synthetic rates were 7.4 ± 2.3 μg/mg proteins per hour. No albumin synthesis was observed in the precision-cut liver slices. Human HepG2 cells produced 7.5 ± 0.8 μg/mg proteins per hour.

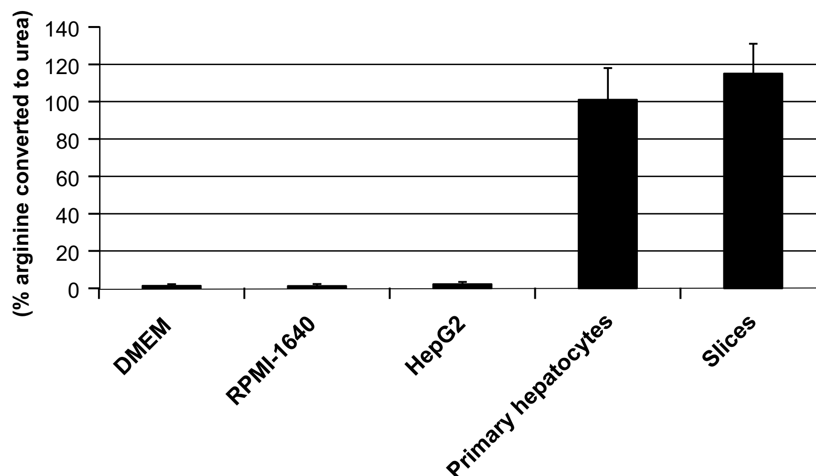

Fig. 1. Percentage of arginine conversion to urea (± standard deviation) in three different hepatocytes cell culture systems after 24 h incubations ($n = 6$). Because different cell culture media were used, the conversion of arginine to urea was expressed as percentage. For concentrations: see Tables 1 and 2. In the primary and liver slice cultures the conversion of arginine to urea is not significantly different from 100%. The conversion of HepG2 cells and control media is not significantly different from 0%.

Table 1
Concentrations of arginine in the media (± standard deviation) and urea in the supernatants after 24 h incubation ($n = 6$)

	Arginine (mM)	Urea (mM)	% Conversion of arginine into urea
DMEM	0.39 ± 0.08	0.02 ± 0.02	1.40 ± 0.88
RPMI-1640	1.1 ± 0.3	0.01 ± 0.01	1.2 ± 1.1
HepG2	1.1 ± 0.3	0.03 ± 0.01	2.3 ± 1.3
Primary cells	0.39 ± 0.08	0.39 ± 0.08	99 ± 19
Precision-cut liver slices	1.1 ± 0.3	1.3 ± 0.2	115 ± 7

HepG2 cells and primary hepatocytes were incubated in DMEM (0.4 mM arginine) and precision-cut liver slices were incubated in RPMI-1640 (1.14 mM arginine). No statistically significant arginine was converted to urea in the HepG2 cell culture. All arginine was converted in both primary hepatocytes and precision-cut liver slices. The percentage of arginine converted to urea is given in the third column, because different media were used.

Table 2
Concentrations of arginine (± standard deviation) and urea in the supernatants after 8 h incubation in the media of the first experiment ($n = 6$)

	Arginine (mM)	Urea (mM)	% Conversion of arginine into urea
Primary hepatocytes	0.38 ± 0.08	0.30 ± 0.09	79 ± 3
Precision-cut liver slices	0.98 ± 0.18	1.10 ± 0.24	115 ± 6

To confirm arginase activity, arginine was added to arginine-depleted media. Again, all arginine was converted to urea in the media of the precision-cut liver slices and 79% of the arginine of the primary hepatocytes. The percentage of arginine converted to urea is given in the third column, because different media were used.

4. Discussion

Arginase (L-arginine ureohydrolase EC 3.5.3.1), which is highly prevalent in hepatocytes (Wu and Morris, 1998), hydrolyses arginine into urea and ornithine (O'Sullivan et al., 1996). It has been suggested as a parameter for liver damage, since arginase can escape from damaged cells into the plasma and its activity is relatively stable (Grasemann et al., 2006; Ikemoto et al., 2001). Hence, when carrying out *in vitro* studies with hepatocytes, cell damage might result in arginase leakage in cell culture media (O'Sullivan et al., 1996; Wu and Morris, 1998).

In this study it was shown that arginase leakage from primary hepatocytes and precision-cut liver slices into the supernatants of the cell cultures has a dramatic effect on the arginine concentration in the media. Arginase depleted the supernatants of arginine, which was completely converted to urea.

The presence of arginase in the supernatants was confirmed by showing its activity in an additional incubation in the arginine-depleted supernatants of the cell cultures. All arginine was converted into urea in the supernatants of the precision-cut liver slices and 79% of arginine was converted to urea in the supernatants of the primary hepatocytes (Table 1).

HepG2 cells are known to be deficient in two essential urea cycle enzymes, namely transcarbamylase and arginase I (Mavri-Damelin et al., 2007). In the incubations in HepG2 cells no arginine was converted to urea. The incubations in HepG2 cells were carried out to show that (i) arginine is not broken down during normal incubations and (ii) arginine is stable during sample preparation for HPLC.

To check hepatocyte viability, albumin was determined in the supernatants of the cell cultures. It was shown that HepG2 cells as well as primary hepatocytes showed significant secretion of albumin, although the supernatant of the latter was depleted of arginine. This may suggest that arginine is not essential for acute phase protein synthesis. In addition, these findings showed that both cell cultures were apparently in good condition. However, the precision-cut liver slice cultures show no significant albumin secretion. So we concluded that, despite the good morphology the liver slices might have been less viable. Nevertheless, the urea concentrations in the supernatants were very high. In conclusion, in the presented experimental setting viable and less viable hepatocyte cell cultures showed high urea concentrations in the supernatants. Therefore, a high urea concentration in the media is not necessarily a valid marker of cell viability.

Two different techniques have been employed so far that use of urea synthesis as viability indicator. The most straightforward method is to determine the urea concentration in the supernatants of hepatocyte cultures and, subsequently, to correlate urea concentration in the media to urea cycle activity (de Kanter et al., 1998; Muller et al., 2004; Sugimachi et al., 2004). However, we discovered that when using this method, results can be biased by the formation of urea by arginase, which has leaked from the cells into the supernatants.

In our opinion, the best method to use urea synthesis as a viability indicator is to incubate the hepatocytes in arginine-free Krebs–Henseleit–Hepes buffer supplemented with ornithine and NH_4Cl. These are the substrates forcing the urea cycle to go through a complete cycle to produce urea. This method has been used successfully by several groups (Day et al., 1999; Sharma et al., 1997). By using this method, leakage of arginase into the buffer will not bias urea production, since transmembrane transport of the intermediates ornithine and citrulline is essential in the urea cycle and the hydrolysis of arginine by arginase is the final and not the rate-limiting step in the cycle.

One study by Maas et al. (2000) used the combination of the two methods by adding ornithine and NH_4Cl to the arginine containing cell culture media. They interpreted their results as significant, however, not being able to discriminate between urea formed from the urea cycle or from arginine cleavage in the media.

In conclusion, when choosing urea production of primary hepatocytes cultures as a viability indicator, one has to take into account that arginase can leak from the cells into the supernatant. This can lead to an overestimation of the viability of the cells, since arginase converts arginine into urea without involvement of the urea cycle. We suggest using an extra incubation in an arginine-free

buffer supplemented with ornithine and NH_4Cl. In addition, arginase leakage can lead to depletion of the supernatant of arginine in primary hepatocytes cell cultures, which might have consequences for experiments studying cellular activities, like, e.g. nitric oxide (NO) production (Durante et al., 2007). Further research is needed to clarify the exact rate of the reaction in order to estimate the magnitude of the effect in relation to cell culture characteristics like acute phase protein synthesis and NO synthesis.

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