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ORIGINAL PAPER

Comparison of Primary and Secondary Rat Astrocyte Cultures Regarding Glucose and Glutathione Metabolism and the Accumulation of Iron Oxide Nanoparticles

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Abstract Astrocyte-rich primary cultures (APCs) are frequently used as a model system for the investigation of properties of brain astrocytes. However, as APCs contain a substantial number of microglial and oligodendroglial cells, biochemical parameters determined for such cultures may at least in part reflect also the presence of the contaminating cell types. To lower the potential contributions of microglial and oligodendroglial cells on properties of the astrocytes in APCs we prepared rat astrocyte-rich secondary cultures (ASCs) by subculturing of APCs and compared these ASCs with APCs regarding basal metabolic parameters, specific enzyme activities and the accumulation of iron oxide nanoparticles. Immunocytochemical characterization revealed that ASCs contained only minute amounts of microglial and oligodendroglial cells. ASCs and APCs did not significantly differ in their specific glucose consumption and lactate production rates, in their specific iron and glutathione contents, in their specific activities of various enzymes involved in glucose and glutathione metabolism nor in their accumulation of iron oxide nanoparticles. Thus, the absence or presence of some contaminating microglial and oligodendroglial cells appears not to substantially modulate the investigated metabolic parameters of astrocyte cultures.

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Introduction

Astrocytes are the most abundant cell type in the brain [1]. Their passive character as "glue" has long been out of date and an increasing number of functions have been reported for astrocytes over the past decades [2]. Since the end feet of astrocytes are in direct contact with the blood capillaries, astrocytes are considered to supply neurons and other glial cell types with nutrients and to maintain the homeostasis of water and ions in brain [3, 4]. Furthermore, astrocytes can modulate the concentration of transmitters by uptake of neuro- and release of gliotransmitters [2, 3]. Astrocytes are considered to be involved in the exchange of energy substrates [5–8] and supply neurons with precursors for glutathione (GSH) synthesis [9, 10]. In addition, astrocytes have been discussed to protect neurons against the toxicity of metals [11, 12] and metal-containing nanoparticles [13].

Astrocyte-rich cultures have frequently been used as a model system for the investigation of astrocyte functions [14]. The majority of studies on cultured astrocytes were performed on astrocyte-rich primary cultures (APCs) that are prepared from cerebral parts of neonatal rodent brain [15]. However, due to the cerebral origin, APCs are often contaminated with a proportion of other glial cell types, mainly by microglial cells [16–18]. In APCs, the presence of contaminating glial cell types could affect the overall specific metabolic properties determined for the culture either by their contribution to the metabolism of the culture and/or by the release of factors that may modulate the metabolic properties of the astrocytes present in the

cultures. Several methods have been described to reduce the number of microglia in astrocyte-rich cultures, such as subculturing of APCs with a low re-seeding cell density, application of microglia toxins, shaking of the cultures to remove the microglial cells present on the top of the astroglial layer or magnetic separation [17, 18].

To test whether contaminating microglial and oligodendroglial cells in APCs affect the basal metabolic properties of the astrocytes, which represent the majority of cells in these cultures, we generated astrocyte-rich secondary cultures (ASCs) by subculturing APCs and confirmed that these ASCs contain only minute numbers of contaminating microglial and oligodendroglial cells. These ASCs were compared with APCs regarding glucose consumption and lactate production, the export of GSH, the specific activities of several important metabolic enzymes and the accumulation of iron oxide nanoparticles (IONPs). The data obtained revealed that ASCs and APCs did not significantly differ in the metabolic parameters investigated, suggesting that the contaminating microglial and oligodendroglial cells in APCs do not affect the investigated metabolic parameters of these cultures.

Materials and Methods

Material

Bovine serum albumin, NADH, NADPH, NAD^+ and $NADP^+$ were from Applichem (Darmstadt, Germany). Fetal calf serum (FCS) and penicillin/streptomycin solution were obtained from Biochrom (Berlin, Germany) and Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Karlsruhe, Germany). All enzymes as well as glutathione (GSH) and glutathione disulfide (GSSG) were obtained from Roche (Mannheim, Germany). Dimercaptosuccinate-coated iron oxide nanoparticles (IONPs) were prepared and characterized as recently described [19]. Mouse anti-rat CD11b was from AbD Serotec (Düsseldorf, Germany). Goat Cy2-conjugated anti-rabbit immunoglobulin (IgG) $F(ab)_2$, goat Cy3-conjugated anti-mouse IgG and donkey anti-sheep IgG conjugated with Cy3 were obtained from Dianova (Hamburg, Germany). Mouse anti-glutamine synthetase (GS) was purchased from BD Transduction Laboratories (Heidelberg, Germany) and rabbit anti-glial fibrillary acidic protein (GFAP) from DakoCytomation (Hamburg, Germany). Sheep anti-myelin associated glycoprotein (MAG) [20] was kindly provided by Dr. Frank Dietz (University of Bremen). Other chemicals of the highest purity available were obtained from Merck (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), Roth (Karlsruhe, Germany) or Janssen (Geel, Belgium). 96-well microtiter plates were purchased from Nunc (Wiesbaden, Germany) and sterile 24-well cell culture plates were from Sarstedt (Nümbrecht, Germany).

Cell Culture

Astrocyte-rich primary cultures (APCs) were prepared from brains of neonatal Wistar rats as described earlier [21, 22]. The harvested cells were seeded into wells of 24 well-plates with or without coverslips (300,000 cells/well) or in 25 cm^2-flasks (3 million cells/flask). The culture medium (DMEM containing 10 % FCS, 25 mM glucose, 1 mM pyruvate, 20 units/mL penicillin G and 20 µg/mL streptomycin sulfate) was renewed every seventh day and the APCs were used for experiments at a culture age of 14–21 days. After 2 weeks in culture, the APCs grown in 25 cm^2-flasks were used to prepare ASCs. Loosely attached cells were detached by hitting the flask, removing the culture medium and washing the attached cells with sterile phosphate buffered saline (PBS; 10 mM potassium phosphate buffer pH 7.4 containing 150 mM sodium chloride). The remaining confluent cell layer was exposed to 1.5 mL 0.25 % (w/v) trypsin in PBS for 7 min at 37 °C. Subsequently, the trypsinization was stopped by addition of 3 mL DMEM with 10 % FCS and the cells were centrifuged (400 g, 5 min, 4 °C). The cell pellet was resuspended in DMEM with 10 % FCS, the cell viability was determined by nigrosine exclusion [23] and 10,000 viable cells were seeded per well of a 24-well plate (with or without coverslips). The cells in ASCs were grown for up to 3 weeks and the culture medium was renewed every seventh day. After 3 weeks in culture, ASCs had reached confluence.

Immunocytochemical Staining

Cells of APCs and ASCs were washed with cold PBS and fixed with 400 µL 3.5 % (w/v) paraformaldehyde in PBS for 10 min at room temperature (RT). Cells were washed thrice with cold PBS in intervals of 5 min prior to application of 400 µL 0.1 % (w/v) glycine in PBS for 10 min at RT and a subsequent permeabilization with 400 µL 0.3 % (w/v) Triton X-100 in 0.1 % glycine for 10 min at RT. After washing three times with PBS, cells which should be stained for GS were incubated for 1 h at RT with 5 % bovine serum albumin in PBS. Cells were washed thrice with PBS and incubated with primary antibodies (rabbit anti-GFAP 1:200, mouse anti-GS 1:200, mouse anti-CD11b 1:100, sheep anti-MAG 1:500) over night at 4 °C, washed and incubated with secondary antibodies (Cy 3-labeled anti-mouse 1:200, Cy 2-labeled anti-rabbit 1:200, Cy 3-labeled anti-sheep 1:500) for 30 min at RT. After incubation with the secondary antibody, nuclei were

counterstained with DAPI (1 μg/mL in PBS) for 5 min at RT. Cells were washed thrice with PBS and dehydrated by an ethanol serial (70–100 %) before embedding in DPX mounting medium. Cellular fluorescence was monitored by an Eclipse TE-2000U fluorescence microscope with a DS-QilMc camera and imaging software NIS-Elements BR (Nikon, Düsseldorf, Germany) using appropriate filter sets for Cy2 (excitation: 465–495 nm, emission: 505–515 nm, dichromatic mirror: 505 nm), Cy3 (excitation: 510–560 nm, emission: 590 nm, dichromatic mirror: 575 nm) and DAPI (excitation: 330–380 nm, emission: 420 nm, dichromatic mirror: 400 nm).

Experimental Incubations

To determine glucose consumption, lactate production and glutathione export from cultured astrocytes, the cells were washed with 1 mL incubation buffer (IB; 20 mM HEPES, 5 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.4 mM KCl, 145 mM NaCl, pH 7.4) and incubated with 1 mL (glutathione export) or 200 μL (lactate release, glucose consumption) IB for up to 6 h at 37 °C. At the indicated time points, the media were collected to determine the extracellular contents of glutathione, lactate and glucose and the cells were washed with 1 mL ice-cold PBS before analysis of cellular protein and glutathione contents.

The accumulation of iron oxide nanoparticles by cells in ASCs and APCs was investigated by incubation for 4 h with 0.75 or 1.5 mM iron as IONPs at 37 or 4 °C. Media were collected for determination of extracellular lactate dehydrogenase (LDH) activity as indicator for a loss of cell viability. The cells were washed with cold PBS and either stored at −20 °C for determination of protein and iron contents or used directly for Perls' staining to visualize cellular iron.

Determination of Metabolic Parameters and Enzyme Activities

The cellular protein content per well was determined by the Lowry method [24] using bovine serum albumin as a standard. Cellular iron contents were quantified by a modification [25] of a previously published colorimetric method in microtiter plates [26]. Cytochemical staining for iron was done as recently described [19, 25]. The amount of total glutathione (GSx = GSH + 2 GSSG) and glutathione disulfide (GSSG) in media samples and cell lysates were determined by the colorimetric Tietze assay [27]. The concentrations of lactate and glucose in media were determined by coupled enzymatic assays as previously described [22, 28, 29].

The specific activities of metabolic enzymes in astrocyte-rich cultures were determined after lysis of the cultures in 200 μL 20 mM potassium phosphate buffer (pH 7.4) containing 1 % (w/v) Triton X-100. The activities of citrate synthase (CS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR) and LDH were determined as previously described [29, 30] and the activities obtained were normalized to the protein content per well to calculate the specific activities.

Presentation of the Data

The data presented were obtained in at least three experiments performed on independently prepared cultures. Results are presented as mean values ± standard deviation. Significance of differences between multiple sets of data was analysed by ANOVA followed by the Bonferroni post hoc-test, while differences between two sets of data were analysed by the *t* test. *P* values >0.05 were considered as not significant. The microscopical images shown are derived from one culture but are representative for at least three experiments performed on independently prepared cultures.

Results

Morphology and Immunocytochemical Characterization of ASCs and APCs

Passaging of the cells in APCs and re-seeding in a cell density of 10,000 cells/well generated within 3 weeks a confluent ASC. The morphology of the cells in ASCs was rather flat giving a low phase contrast (Fig. 1a, c). In contrast, cells in APCs were more round and some had long processes (Fig. 1b, d). In addition, the contrast-rich cells on top of the basal cell layer in APCs (Fig. 1b, d) were not detectable anymore in the ASCs (Fig. 1a, c). Immunocytochemical analysis for the presence of marker proteins of different glial cell types revealed that both ASCs and APCs were strongly enriched in astrocytes, as deduced from the large number of cells that expressed the astrocyte marker proteins GFAP and GS (Fig. 2a, b, c, d). However, the staining intensity and the staining pattern for GFAP differed between the two types of cultures. While in APCs the majority of cells were strongly stained for GFAP (Fig. 2b), most cells of ASCs were only weakly stained for GFAP (Fig. 2a; Table 1). However, some patches of cells in ASCs were also positive for both GFAP and GS (Fig. 2a, c), while such patches were not observed in APCs (Fig. 2b, d). Figure 3 shows an example for the immunocytochemical discrimination in ASCs between strongly and weakly GFAP-positive astrocytes. Cells strongly positive for GFAP revealed an

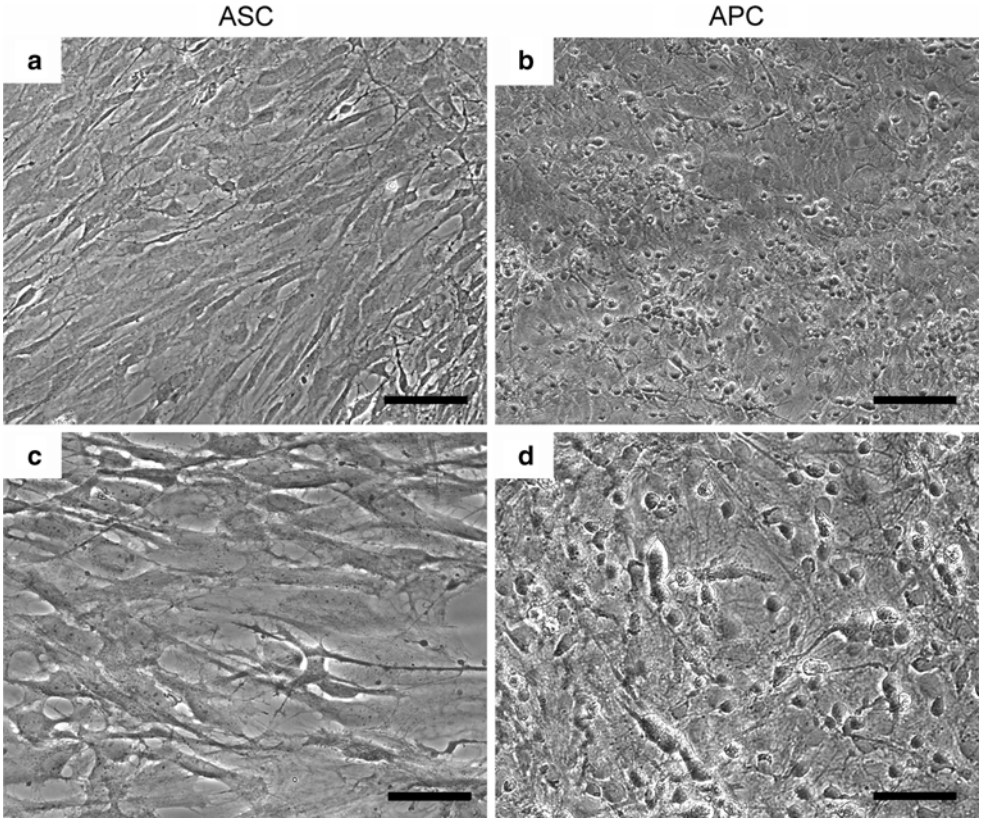

Fig. 1 Phase contrast images of ASCs and APCs. The *scale bars* in **a** and **b** represent 100 μm, the *bars* in **c** and **d** represent 50 μm

intensive fluorescence signal after a microscopical exposure time of 800 ms (Fig. 3a), while under such exposure conditions weakly stained GFAP-positive cells were not detectable. Clear signals for cells that were weakly stained for GFAP were obtained after an exposure time of 2 s (Fig. 3b). Omission of the GFAP antibody revealed a very low unspecific background staining for both exposure times (Fig. 3c, d).

Presence of microglial cells and oligodendrocytes was investigated by staining of ASCs and APCs for CD11b (Fig. 2e, f) and MAG (Fig. 2g, h). Immunocytochemical staining of APCs revealed a large number of cells that were strongly GFAP-positive (Fig. 2b), some GS-positive cells (Fig. 2d) and a large number of small fluorescent elements that were positive after staining for the microglial marker CD11b (Fig. 2f) and the oligodendroglial marker MAG (Fig. 2h). In contrast, ASCs contained predominately cells that were weakly stained for GFAP and hardly any cells that were positive for the microglial marker CD11b (around 1 %) and the oligodendroglial marker MAG (around 3 %) (Fig. 2e, g; Table 1). We also tried to quantify in APCs the number of cells that were strongly GFAP-positive (65 ± 24 %), weakly GFAP-positive (3 ± 5 %), CD11b-positive (26 ± 4 %) and MAG-positive (8 ± 4 %). However, with our equipment it was not possible to reliably connect in the confluent APCs a given nucleus with all stained microglial or oligodendroglial elements that belong to the respective cell. Thus, the results obtained in our attempt to quantify in APCs the number of CD11b-positive cells and MAG-positive cells are likely to overestimate the real number of microglial and oligodendroglial cells.

Protein Content and Viability of Astroglia-Rich Cultures

To investigate whether the presence or almost absence of contaminating glial cells may affect overall basal metabolic parameters of the cultures, we compared ASCs and APCs regarding their glucose and glutathione metabolism as well as for their ability to accumulate IONPs. The protein content of the 3 weeks old ASCs (114 ± 26 μg/well) was almost identical to that of APCs (109 ± 5 μg/well). In contrast, the protein content of 2 weeks old ASCs (68 ± 15 μg/well) was significantly lower ($p < 0.01$) than that of 3 weeks old ASCs (114 ± 26 μg/well), demonstrating that ASCs have to be cultured for 3 weeks before they have a protein content that is almost identical to that of APCs.

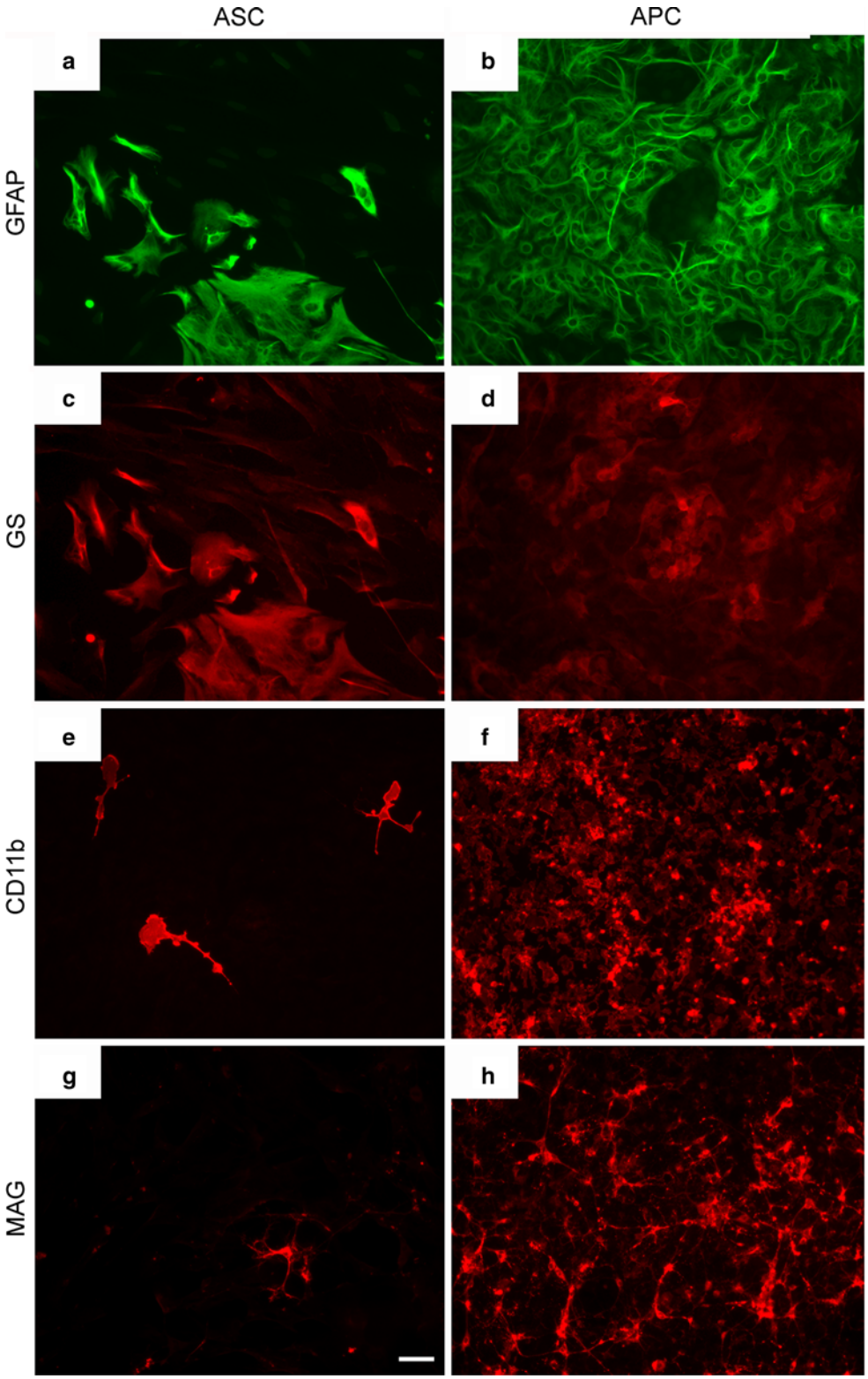

Fig. 2 Immunocytochemical staining of a 21 d-old ASC and a 14 d-old APC. Astrocyte cultures were stained for the astrocytic marker proteins GFAP (**a, b**) and GS (**c, d**), for the microglial marker protein CD11b (**e, f**) or for the oligodendrocyte marker protein MAG (**g, h**). The *scale bar* in (**g**) represents 50 μm and applies to all panels

Table 1 Immunocytochemical characterization of ASCs

Immunostaining for	Number of positive cells (%)
GFAP (strong staining)	25 ± 38
GFAP (weak staining)	70 ± 40
CD11b	1 ± 2
MAG	3 ± 5

Three individually prepared ASCs were immunocytochemically stained for the presence of GFAP, CD11b or MAG and the number of positive cells were counted and compared with the total number of cells (determined as number of nuclei present which were stained with DAPI). For each culture the cells in 5–10 microscopic fields (68,735 μm^2) were counted. A total number of 8,410 cells in ASCs was identified by immunocytochemical staining. Depending on the intensity of the GFAP staining, the cells were discriminated as strongly or weakly GFAP-positive as depicted in Fig. 3

For determining the glucose and glutathione metabolism of the different types of astrocyte-rich cultures, the cells were incubated in IB for up to 6 h. Microscopical inspection revealed that during this time frame, no obvious detachment of cells nor any loss in cell viability were observed (data not shown) which was confirmed by the almost constant protein content (Fig. 4a) and by the absence of any significant increase in the extracellular LDH activity (Fig. 4b) during the incubation of ASCs or APCs.

Glucose Metabolism of ASCs and APCs

Incubation of ASCs or APCs in glucose-containing medium caused an almost linear increase in the extracellular concentration of lactate (Fig. 4c) and an almost linear decrease in extracellular glucose concentration (data not shown) which is reflected by the calculated almost linear increases in cellular glucose consumption (Fig. 4d). The almost linear increases in extracellular lactate accumulation and in glucose consumption within the first 4 h of incubation were used to calculate the rates of lactate production and glucose consumption for both ASCs and APCs. The specific lactate production rates of 0.8 ± 0.2 µmol/(h mg) (ASCs) and 1.0 ± 0.2 µmol/(h mg) (APCs) and the specific glucose consumption rates of 0.5 ± 0.2 µmol/(h mg) (ASCs) and 0.5 ± 0.1 µmol/(h mg) (APCs) did not differ significantly (Table 2). Also the ratio of the lactate production rate to the glucose consumption rate of ASCs (1.8 ± 0.2) and APCs (1.9 ± 0.3) was almost identical for both culture types. In addition, the specific activities of some key enzymes in glucose and energy metabolism, i.e.,

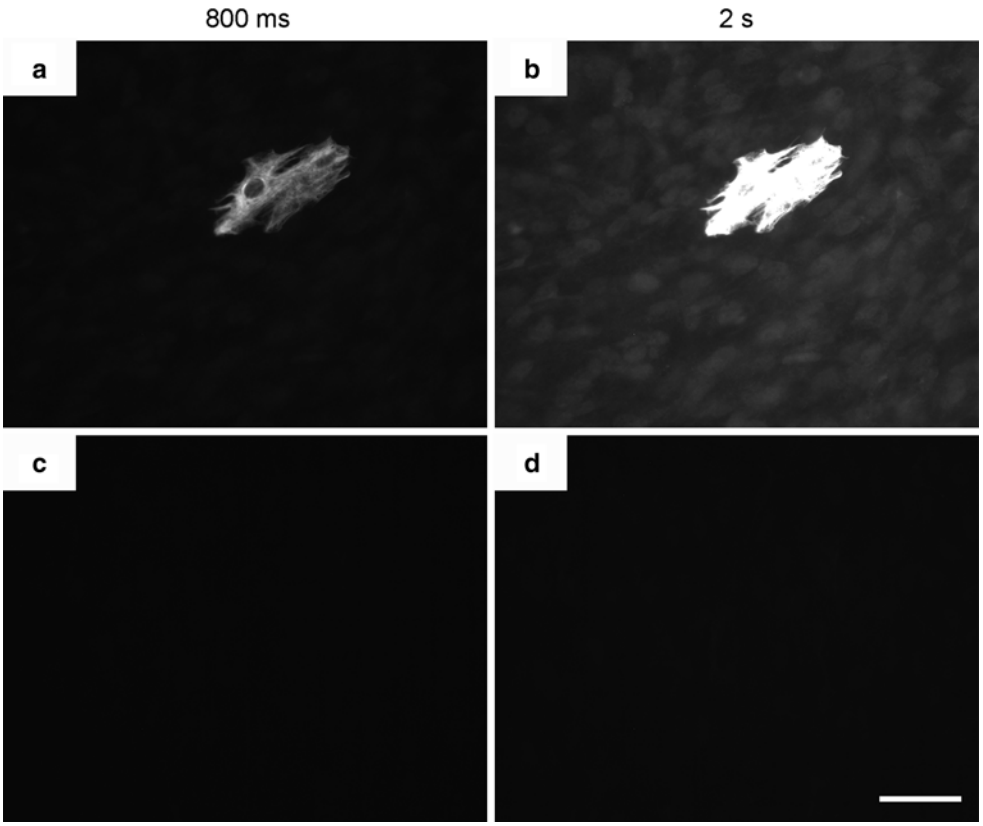

Fig. 3 Identification of cells in ASCs that are strongly and weakly GFAP-positive. ASCs were immunocytochemically stained for GFAP and monitored on the fluorescence microscope by either a short (800 ms; **a, c**) or a long (2 s; **b, d**) exposure time. To identify the level of unspecific background fluorescence, the anti-GFAP antibody was omitted in the staining procedure (**c, d**)

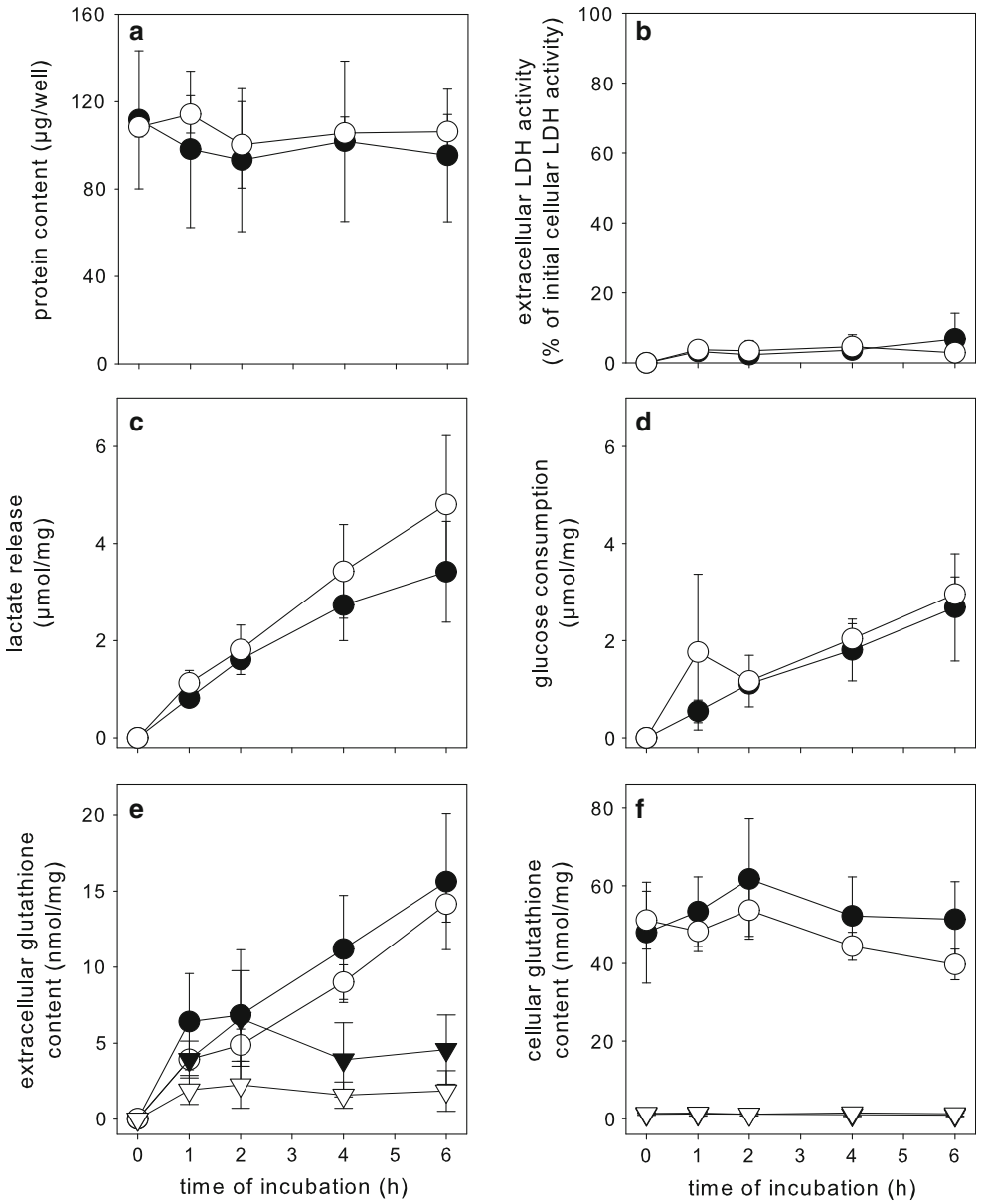

Fig. 4 Glucose and glutathione metabolism of ASCs and APCs. ASCs (*black symbols*) and APCs (*white symbols*) were incubated for up to 6 h at 37 °C and the cellular protein content (**a**), the extracellular LDH activity (**b**), the specific extracellular lactate content (**c**), the specific glucose consumption (**d**), as well as the extracellular (**e**) and cellular (**f**) contents of GSx (*circles*) and GSSG (*triangles*) were determined. Significant differences between the data obtained for ASCs and APCs (n = 3) were not observed

G6PDH, GAPDH, LDH and CS, did not differ significantly in ASCs and APCs (Table 3).

Glutathione Metabolism of ASCs and APCs

Quantification of the basal GSx contents of untreated ASCs and APCs revealed that these cultures contained almost identical levels of GSx with 48 ± 13 and 51 ± 7 nmol GSx/mg protein, respectively. Only minute amounts of GSSG contributed to these GSx contents as the specific GSSG contents amounted to only 1.2 ± 0.3 (ASCs) and 1.4 ± 0.1 nmol GSx/mg (APCs).

Incubation of ASCs or APCs in IB for up to 6 h caused an almost linear increase in the extracellular contents of GSx (Fig. 4e) which was accompanied with a small, but not significant loss in cellular GSx (Fig. 4f). Some of the extracellular GSx accounted for GSSG, while in the cells the GSSG levels were in the range of the detection limit of the method applied (Fig. 4f). Calculation of the rates of extracellular accumulation of GSx revealed that ASCs

Table 2 Rates of glucose consumption, lactate release and extracellular glutathione accumulation in ASCs and APCs

	ASCs	APCs
Lactate release rate (μmol/(h mg))	0.8 ± 0.2	1.0 ± 0.2
Glucose consumption rate (μmol/(h mg))	0.5 ± 0.2	0.5 ± 0.1
Ratio of lactate release to glucose consumption	1.8 ± 0.2	1.9 ± 0.3
GSH export rate (nmol/(h mg))	2.6 ± 0.8	2.4 ± 0.1

The almost linear increases in glucose consumption, lactate release and extracellular GSx accumulation during the first 4 h of incubation (Fig. 4) were used to calculate the specific rates for glucose consumption, lactate production and GSH export. Significant differences between the data obtained for ASCs and APCs were not observed. The data are derived from experiments performed on three individually prepared cultures of ASCs and APCs

Table 3 Specific enzyme activities determined for ASCs or APCs

	Enzyme activity (nmol/(min · mg protein))			
	ASCs	n	APCs	n
Glyceraldehyde-3-phosphate dehydrogenase	189 ± 63	3	158 ± 53	3
Lactate dehydrogenase	1,080 ± 194	5	1,108 ± 126	7
Citrate synthase	65 ± 4	3	75 ± 6	5
Glutathione reductase	7 ± 3	4	8 ± 2	5
Glucose-6-phosphate dehydrogenase	23 ± 2	3	25 ± 4	5

The data are derived from experiments performed on n individually prepared cultures of ASCs and APCs. Significant differences between the data obtained for ASCs and APCs were not observed

[2.6 ± 0.8 nmol/(h mg)] and APCs [2.4 ± 0.1 nmol/(h mg)] had almost identical rates of GSH export (Table 2). In addition, the specific activity of GR, which reduces GSSG to GSH, was almost identical for both types of astrocyte cultures (Table 3).

Iron Content and Accumulation of Iron Oxide Nanoparticles by ASCs and APCs

Untreated ASCs and APCs had almost identical basal iron contents of 8 ± 4 and 10 ± 2 nmol/mg, respectively. Perls' staining of untreated APCs for iron revealed that a few cells in these cultures were strongly positive for iron (Fig. 5b), while such cells were not detectable in ASCs (Fig. 5a). The basal iron contents of ASCs or APCs were not significantly elevated during incubation for 4 h at 37 °C or at 4 °C in the absence of IONPs (Table 4). In contrast, presence of 0.75 or 1.5 mM iron as IONPs during the incubation at 37 °C strongly increased the specific cellular iron content of both ASCs and APCs to values of around 1,500 and 2,500 nmol iron/mg protein, respectively (Table 4). The IONP-treated cells displayed a strong staining for iron which did not differ between ASCs and APCs (Fig. 5c, d). Compared to the 37 °C incubation condition, the cellular iron accumulation was significantly lowered by around 50–60 %, if ASCs or APCs had been incubated with IONPs at 4 °C (Table 4). For these conditions, only a low intensity of the Perls' iron staining was observed for ASCs (Fig. 5e), while some Perls'-positive cells were found in APCs that had been exposed to IONPs at 4 °C (Fig. 5f). None of these incubation conditions did lower the cell viability as demonstrated by the absence of a significant increase in extracellular LDH activity and by the almost constant cellular protein content compared to the respective control incubations (0 μM) (Table 4).

Discussion

Astrocyte-rich primary cultures are a frequently used model system to investigate properties and functions of brain astrocytes [14, 15, 22], since these cultures are enriched for astrocytes. However, APCs contain also other types of glial cells which contribute to and/or may affect the basal metabolic properties determined for the cultures. To address this question we prepared and immunocytochemically characterized ASCs and compared their basal metabolic parameters and properties with those of APCs.

Immunocytochemical staining of APCs confirmed the presence of microglial cells and oligodendrocytes in cultures prepared by our [21, 22, 31] or other protocols [32, 33]. Subculturing and seeding of a low number of viable cells to obtain ASCs lowered the number of microglia and oligodendrocytes substantially, confirming literature data [34, 35]. Likely reason for the enrichment in astrocytes in ASCs is that the subculturing prevents contact between the contaminating cells and/or that certain growth factors are missing which would be required for the proliferation of the contaminating cells [35].

In ASCs, the morphology of astrocytes and the expression of astrocyte marker proteins were different to those observed for APCs. The majority of the cells in ASCs were only weakly GFAP-positive, whereas most cells in APCs were strongly stained for GFAP. Furthermore, the GS staining pattern of astrocytes in rat ASCs was similar to their GFAP-staining, while in rat APCs mainly the GFAP-negative astrocytes were positive for GS. These data obtained for rat cultures contrast literature data observed for APCs [36] and ASCs [37] from mouse brain where most astrocytes express both GFAP and GS. Differences in the expression of GFAP and/or GS in cultured astrocytes may reflect the known heterogeneity of astrocytes in brain [38, 39] and/or an enrichment of certain subtypes of astrocytes during culturing and passaging. Furthermore, the

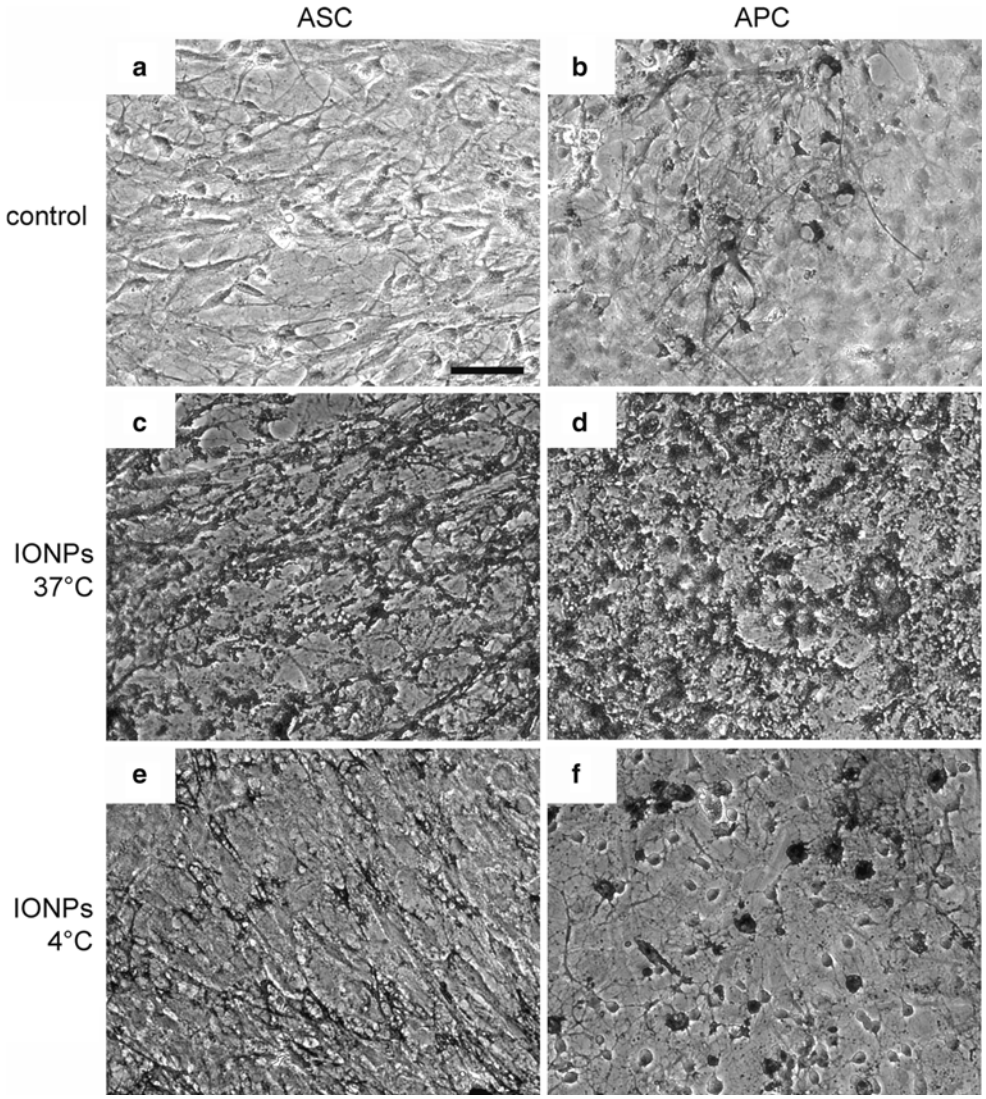

Fig. 5 Perls' iron staining of IONP-treated ASCs and APCs. ASCs or APCs were incubated for 4 h without (**a, b**) or with (**c, d**) 1.5 mM iron as IONPs at 37 °C (**a–d**) or with 1.5 mM iron as IONPs at 4 °C (**e, f**), fixed and stained for iron. The *scale bar* in panel **a** represents 50 μm and applies to all panels

Table 4 IONP accumulation by ASCs and APCs

		Iron content (nmol/mg protein)		Protein content (μg/well)		LDH release (% of initial)	
[IONP] (μM)	T (°C)	ASCs	APCs	ASCs	APCs	ASCs	APCs
0	37	17 ± 9	23 ± 6	110 ± 43	99 ± 7	7 ± 3	8 ± 1
0	4	16 ± 7	27 ± 13	131 ± 22	116 ± 32	9 ± 9	5 ± 4
750	37	1,550 ± 708	1,276 ± 268	138 ± 19	114 ± 7	8 ± 5	8 ± 5
750	4	527 ± 181	691 ± 117*	148 ± 25	119 ± 15	3 ± 5	1 ± 1
1,500	37	2,765 ± 1,160	2,265 ± 487	123 ± 19	117 ± 7	3 ± 3	2 ± 3
1,500	4	892 ± 117*	1,042 ± 75***	131 ± 13	123 ± 16	6 ± 6	6 ± 3

ASCs or APCs were incubated without (0 μM) or with 750 or 1,500 μM iron as IONPs for 4 h at the temperatures (T) of 37 or 4 °C and the specific cellular iron content, the protein content per well and the extracellular LDH activity (as % of the initial cellular LDH activity) were determined. Asterisks indicate significant differences between values obtained for incubations of one type of astrocyte culture at 37 and 4 °C (* $p < 0.05$, *** $p < 0.001$). Significant differences between data obtained for ASCs and APCs were not observed. The data are derived from experiments performed on three individually prepared cultures of ASCs and APCs

expression of astrocytic markers is known to be affected by subculturing, although the literature data are not consistent as subculturing has been reported to increase [40], not affect [37] or decrease [41] GFAP protein levels. This discrepancy may be a consequence of differences in the culturing conditions which are known to strongly affect the expression of cell type specific markers [42–44]. The low expression of GFAP in the astrocytes of ASCs may be a direct consequence of the low number of microglial cells in these cultures, as the culture medium of ASCs is likely to be deprived of microglia-derived factors which have been reported to induce GFAP expression in astrocytes [45, 46]. The low GFAP expression and/or absence of non-astrocytic cell types may also contribute to the flat and contrast-low appearance of astrocytes in the ASCs, contrasting the morphology of the phase-dark and process bearing astrocytes in APCs [47].

Absence or presence of a substantial amount of contaminating microglial cells and oligodendrocytes may affect metabolic parameters that are investigated for astrocyte-rich cultures as the contaminant cell types may differ in their metabolism from astrocytes, thereby affecting the overall parameters determined for the sum of all cells in a given culture. In addition, contact of astrocytes to other cell types in the cultures investigated as well as compounds released by these contaminating cells may activate or suppress metabolic processes in astrocytes. To address such questions we compared APCs with ASCs concerning basal metabolic parameters such as the release of glutathione and lactate as well as the accumulation of IONPs. Remarkably, ASCs and APCs did not differ significantly in specific enzyme activities, specific metabolite levels, glycolytic flux, GSH export or in the accumulation of IONPs, clearly demonstrating that removal of most of the contaminating microglial and oligodendroglial cells from the APCs by generating ASCs did not affect the overall metabolic parameters investigated. These data are consistent with recent findings showing similar metabolism of amino acids in primary and passaged mice astrocyte cultures [37].

Cultured astrocytes are known to have a high glycolytic capacity [48–50]. The data obtained here for glucose consumption and lactate release by ASCs or APCs and the high ratio of lactate release to glucose consumption are similar to those previously reported for cultured astrocytes [51, 52] though lower ratios were also observed for astroglial cultures [53]. The almost identical specific activities of the enzyme GAPDH, G6PDH and LDH in ASCs and APCs support the view that the basal glucose metabolism of these cultures does not differ. The specific activities of GAPDH, LDH and CS determined here were lower as described previously by us [29, 30, 54] or others [55–57], which may be a consequence of the culturing and/or lysis conditions, as we have used for our present study a detergent to lyse the cells for enzyme activity assays.

Astrocytes play an important role in the GSH metabolism of the brain and supply precursors for GSH to neurons in a process that is initiated by GSH export [9, 10, 58]. The basal GSx content of ASCs and APCs determined here (around 55 nmol/mg protein) is similar to values reported previously for such cultures [59, 60]. The very low cellular GSSG content in both APCs and ASCs revealed that the cells in these cultures do not suffer from severe oxidative stress [9]. The high potential of both ASCs and APCs to efficiently maintain low cellular GSSG levels is consistent with the almost identical specific activities of GR, the enzyme which continuously reduces GSSG to GSH in a NADPH-dependent reaction [61], and of G6PDH, the rate limiting enzyme of the NADPH-providing pentose phosphate pathway [62]. The rates of GSH export from ASCs and APCs of around 2.5 nmol/(h mg protein) is similar to literature data for cultured rat or mouse astrocytes [60, 63]. The transporter predominantly responsible for the observed basal GSH export in ASCs and APCs is most likely the multidrug resistance protein 1, as this transporter has been reported to be the primary mediator of GSH export from rat and mouse astrocyte cultures [63, 64].

Astrocytes are considered to have important functions in the distribution of iron in the brain [11] and in the handling of iron oxide nanoparticles [13]. The basal iron contents of ASCs and APCs (8 ± 4 and 10 ± 2 nmol/mg protein, respectively) did not differ from each other and were similar to those previously reported for astrocyte cultures [19, 26, 65]. Thus, the presence of contaminating oligodendrocytes and microglia which have higher iron contents as astrocytes [66–70] had no influence on the basal cellular iron content of these cultures, probably due to their in general smaller size in comparison to astrocytes. However, cytochemical iron staining revealed for untreated APCs some cells that were strongly iron-positive which may just be microglial or oligodendroglial cells as these cells contain high amounts of iron [66–70]. This view is supported by the observation that such iron-positive cells were not found in untreated ASCs.

On application of IONPs, both ASCs and APCs efficiently accumulated the particles in a temperature dependent process, confirming literature data for APCs [19, 71]. The accumulation of IONPs at 37 °C was similarly strong in ASCs and APCs. Although among the different types of brain cells especially microglia are considered to accumulate large amounts of magnetic IONPs [72–74], no difference in IONP accumulation was observed between the microglia-containing APCs and the ASCs that hardly contained microglial cells. These results suggest that the high amounts of iron found in IONP-treated ASCs and APCs reflect predominately the accumulation of IONPs by

astrocytes and that contaminating microglial and oligodendroglial cells in APCs do not overproportional contribute to the overall IONP accumulation in APCs.

In summary, the preparation of rat ASCs by subculturing of APCs resulted in a strong enrichment of astrocytes and a substantial decrease in number of contaminating other glial cell types. Compared to APCs, subculturing of astrocytes to generate ASCs affected the morphology of astrocytes and the expression of the astrocytic markers GFAP and GS, but did not alter the basal metabolic parameters investigated, including glycolytic flux, GSH export and the accumulation of IONPs. Thus, the presence of a moderate number of contaminating glial cells in APCs appears not to substantially affect the contribution of astrocytes in the metabolic pathways investigated. Potential differences in metabolic parameters in ASCs and APCs appear to be in the same range as differences between individual preparations of either type of astrocyte-rich culture.

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Conflict of interest The authors declare that they have no conflict of interest.

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