Astrocytes enhance radical defence in capillary endothelial cells constituting the blood-brain barrier

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Abstract Astrocytes (AC) induce blood-brain barrier (BBB) properties in brain endothelial cells (EC). As antioxidative activity (AOA) is assumed to be a BBB characteristic, we tested whether AC improve AOA of EC. Monocultivated AC showed higher AOA [manganese superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (GPx)] than EC. Cocultivation elevated AOA in EC (MnSOD, CuZnSOD, Cat, GPx), and AC (MnSOD, CuZnSOD, GPx). Hypoxia increased radical-induced membrane lipid peroxidation in monocultivated, but not in cocultivated EC. Thus, EC/AC cocultivation intensifies AOA in both cell types, protects the EC, and therefore, the BBB against oxidative stress. The high AOA is regarded as an essential property of the BBB, which is induced by AC.

Key words: Blood-brain barrier; Free radical; Antioxidative defence enzyme; Hypoxia/reoxygenation; Brain capillary endothelial cell; Astrocyte

1. Introduction

Brain microvascular endothelial cells (EC) form the blood-brain barrier. Astrocytes (AC) ensheath vascular cylinders and induce blood-brain barrier properties in EC, such as tight junctions [1]. Reactive oxygen species, i.e. $^{\bullet}O_2^-$, $^{\bullet}OH$, H_2O_2, $ONOO^-$, cause lipid peroxidation [2] resulting in membrane disturbances [3,4] and can open the blood-brain barrier [5,6]. Reactive oxygen species are continuously generated in the vascular system [7] and in EC [5], which is intensified during cerebral ischemia/reperfusion [5] or Alzheimer's disease [8,9]. Therefore, high activities of antioxidatively acting enzymes in brain microvessels have to be considered an essential property of the blood-brain barrier [5]. Cytosolic copper-zinc and mitochondrial manganese superoxide dismutases (SOD; EC 1.15.1.1) remove $^{\bullet}O_2^-$. Catalase (Cat; EC 1.11.1.6) decomposes H_2O_2. Glutathione peroxidase (GPx; EC 1.11.1.9) eliminates lipid peroxides and H_2O_2 by oxidizing glutathione. Because of a relatively high antioxidative capability [10] AC can protect neurons from oxidative stress [11]. However, the influence of AC on the antioxidative potential of EC has not been thoroughly examined yet. During hypoxia/reoxygenation, the tightness of the EC monolayer is preserved longer in coculture with AC than in an EC monolayer in monoculture which becomes leaky earlier [1].

Because reactive oxygen species increase the permeability of the EC monolayer, protection of EC by a higher antioxidant capacity of AC is assumed. Therefore, this study compares the antioxidative activity of EC and AC. Since AC can induce blood-brain barrier properties in EC, an increase in the antioxidative potential of EC by cocultivation with AC is hypothesized. To test this hypothesis, the activities of enzymes involved in the defence of reactive oxygen species and radical-induced lipid peroxidation were studied in monocultivated and cocultivated EC and AC. Moreover, the influence of hypoxia/reoxygenation accompanied by the release of reactive oxygen species on the radical-induced peroxidation of membrane phospholipid was investigated.

2. Materials and methods

2.1. Preparation and cultivation of cells

Cloned immortalized rat brain endothelial cells (RBE4, passage 43–55, kindly supplied by Prof. Couraud, Paris, France), primary EC and AC from Wistar rats were grown according to [6] in 95% air/5% CO_2. Monocultures of EC were cultivated on 6-well plates (Falcon) coated with rat tail collagen I (Sigma). In coculture, EC were cultivated on filter inserts (0.45 μm pore size, 1.6×10^6 pores/cm^2, Falcon, coated with rat tail collagen I). AC were seeded on the bottom of 6-well plates containing the filters. Growth factor was omitted in the culture medium of EC. EC expressed angiotensin converting enzyme, factor VIII, alkaline phosphatase and γ-glutamyltranspeptidase, which were intensified by cocultivation.

2.2. Hypoxia/reoxygenation

Confluent monolayers were washed with phosphate buffered solution (with Ca^{2+}, Mg^{2+}, glucose-free, pH 7.2; Biochrom). 1 ml/well phosphate buffered solution saturated with Nelson gas (95% N_2/5% CO_2) was added and gassed with Nelson gas for 2 h at 37°C resulting in 3% O_2 in the incubation solution (hypoxia). During reoxygenation, cells were gassed with Carbogen (95% O_2/5% CO_2) for 0.5 h (for enzyme detection) or 1 h (for malondialdehyde). Cells placed in an incubator served as control [12].

2.3. Biochemical measurements, viability and statistical analysis

Cells were scraped off in ice-cold H_2O (Cat, SOD, GPx, malondialdehyde) or in 5% sulfosalicylic acid (glutathione) and homogenized (except malondialdehyde samples) by sonication on ice (3×5 s with breaks of 10 s). Activity of Cat was estimated according to [13] and expressed as k in mg^{-1} min^{-1} (first-order rate constant of the reaction). Glutathione content was determined according to [14]; reduced (GSH) and oxidized (GSSG) glutathione were measured separately in the absence (total glutathione) or after addition of 2-vinylpyridine (for GSSG), using calibration standards. Activities of GPx and SOD were determined using test kits (Randox, RS 506 and SD 125). GPx activity was measured at 37°C as reduction of cumene hydroperoxide (coupled enzymatic assay with glutathione reductase) resulting in NADPH oxidation, which was followed at 340 nm. Activity was calculated in U g^{-1}. For SOD measurement, xanthine and xanthine oxidase were used generating superoxide radicals which react with 2-[4-iodophenyl]-3-[4-nitrophenol]-5-phenyltetrazolium chloride to form a red dye (505 nm). The activity was calculated as the degree of inhibition of

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this reaction at 37°C, using a standard curve for calibration. Activity was expressed as U mg^{-1}. MnSOD was determined after addition of 1 mM KCN inhibiting CuZnSOD specifically. Content of malondialdehyde was ascertained according to [15,16] using HPLC (Shimadzu LC-10A chromatograph with a RF-10A fluorescence detector) and malondialdehyde bis-[diethylacetal] (Merck) as standard. Under control culture conditions, malondialdehyde content was 0.18 ± 0.04 nmol mg^{-1} in RBE4 and 0.63 ± 0.09 nmol mg^{-1} in AC. Protein was determined by the Lowry method (test kit, Sigma). Cell viability was detected using the neutral red assay (50 μg ml^{-1} cultivation medium, containing 2% FBS, 2 h at 37°C). After washing, the absorbed dye was extracted (50% ethanol/1% glacial acetic acid), and measured (540 nm) [16]. Data represent mean ± S.D. Significance values were calculated by Mann-Whitney rank sum test or Kruskal-Wallis one way ANOVA on ranks followed by Student-Newman-Keuls test.

3. Results

The cell clone RBE4 expressed an antioxidative defence similar to primary EC. Activities of Cat, GPx, content of GSH and GSSG did not differ significantly between primary EC (2.6 ± 0.8 min^{-1} mg^{-1}, 28 ± 16 U g^{-1}, 89 ± 21 nmol mg^{-1}, 1.9 ± 2.6 nmol mg^{-1}; $n = 5$–8) and RBE4 (2.7 ± 0.2 min^{-1} mg^{-1}, 27 ± 9 U g^{-1}, 76 ± 10 nmol mg^{-1}, 0.35 ± 0.2 nmol mg^{-1}; $n = 6$–10). RBE4 possessed sufficient SOD activity (3.2 ± 0.2 U mg^{-1}, $n = 10$) compared to primary EC (1.9 ± 0.6 U mg^{-1}, $n = 6$; $P < 0.001$). Thus, RBE4 are suitable for studying antioxidative defence in brain endothelial cells and were used for all other experiments.

In monoculture, AC showed a higher antioxidative potential than EC (Fig. 1). In AC, the activities of MnSOD, Cat and GPx were $229 \pm 68\%$, $164 \pm 40\%$ and $449 \pm 89\%$, respectively, compared to those in EC ($P < 0.003$). GSSG was below the detection limit in AC but detectable in EC. CuZnSOD activity and GSH content did not differ significantly between EC and AC. In coculture, EC and AC expressed significantly higher activities compared to the respective monoculture except Cat in AC, which was not significantly changed (Fig. 1). Activity of CuZnSOD was only slightly elevated in EC after cocultivation compared to the monocultivation (to $128 \pm 10\%$). The activities of CuZnSOD and GPx in AC were $203 \pm 9\%$ and $231 \pm 22\%$, respectively, the activities of Cat and GPx in EC were $219 \pm 90\%$ and $199 \pm 40\%$, compared to the respective monoculture. The activity of MnSOD increased in AC to $698 \pm 193\%$, in EC to $993 \pm 196\%$. The content of GSH decreased in both EC and AC, but the difference was significant in EC only. GSSG was detectable neither in EC nor in AC when cocultivated.

During hypoxia and reoxygenation, the total antioxidative capability did not change substantially ($n = 8$, if not stated otherwise; vs. the respective control). In monocultivated EC, activities of Cat, GPx and CuZnSOD decreased after hypoxia (to $78 \pm 19\%$, $73 \pm 15\%$, $78 \pm 10\%$; $P < 0.05$ for GPx, CuZnSOD), whereas MnSOD activity was elevated to $122 \pm 21\%$ ($P < 0.05$). After reoxygenation, activities of Cat, CuZnSOD and MnSOD exhibited control levels, GPx differed from the control ($88 \pm 13\%$; $P < 0.05$). Monocultivated AC were less sensitive to hypoxia than EC: only Cat activity declined (to $79 \pm 9\%$; $P < 0.05$), GPx ($n = 4$) and CuZnSOD ($n = 6$) remained unchanged. MnSOD activity increased to $185 \pm 63\%$ ($P < 0.05$). During reoxygenation of AC, Cat activity remained diminished ($80 \pm 9\%$; $P < 0.05$), GPx remained unchanged, whereas CuZnSOD fell to $85 \pm 4\%$ ($P < 0.05$). MnSOD activity tended to return to control level

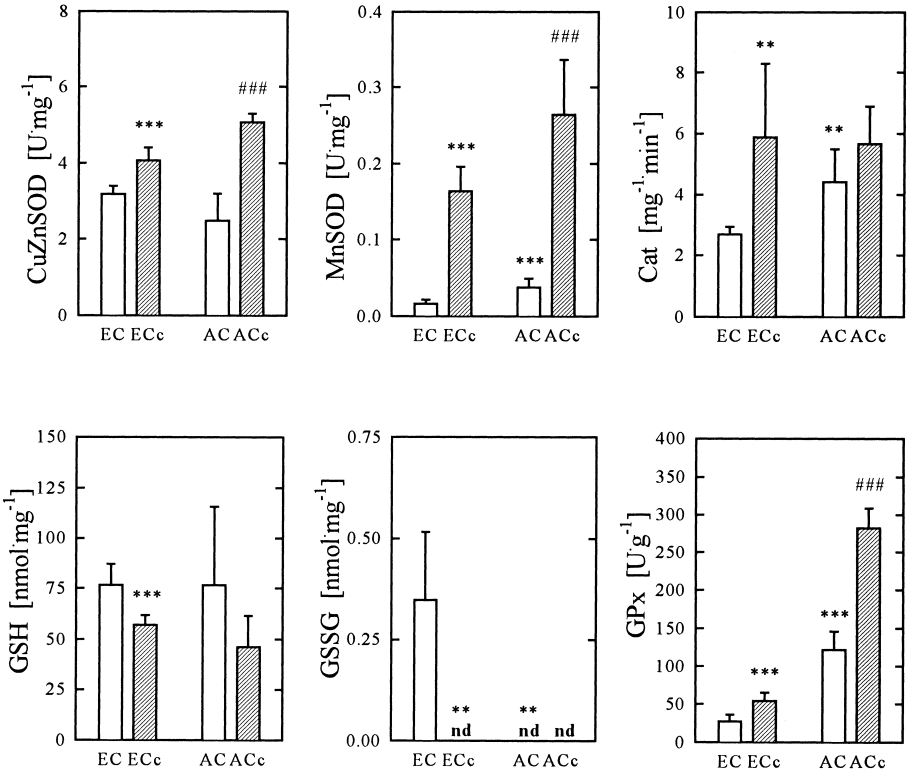

Fig. 1. Activity of CuZnSOD, MnSOD, Cat, GPx and content of GSH, GSSG in brain EC and AC, in monoculture and in coculture (ECc, ACc). Mean ± S.D.; CuZnSOD/MnSOD/GSH/GPx, $n = 10$; Cat/GSSG, $n = 6$. ***$P < 0.001$, **$P < 0.01$ in comparison to EC; ###$P < 0.001$ in comparison to AC; 'nd' means not detectable (detection limit for glutathione 0.02 nmol mg^{-1}).

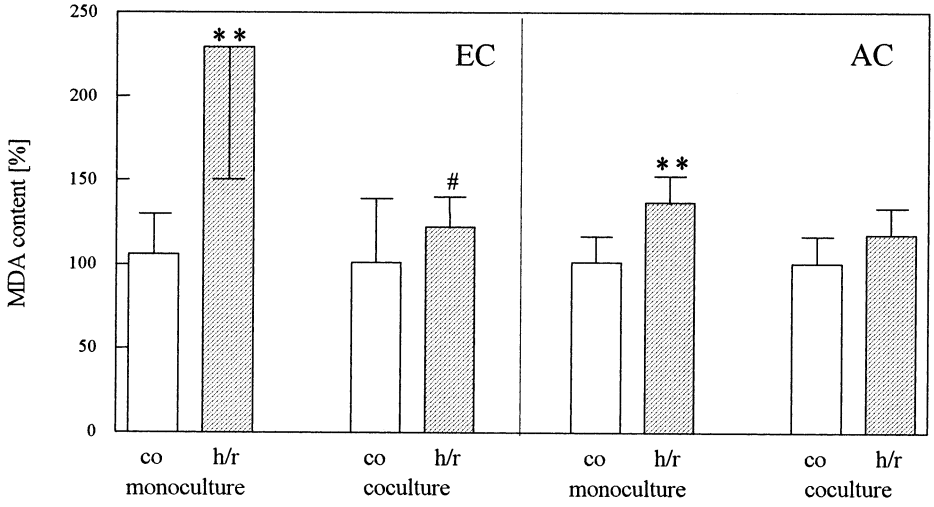

Fig. 2. Changes of malondialdehyde (MDA) content in brain EC and AC after 2 h hypoxia followed by 1 h reoxygenation (h/r) in comparison to control (co). Mean ± SD; $n = 6$ in each group. $^{**}P < 0.01$ in comparison to the respective control, monoculture; $\#P < 0.05$ in comparison to the respective control, coculture.

(121 ± 33%; $P < 0.05$). Cocultivated EC were less sensitive than monocultivated EC: all enzyme activities did not differ significantly from the control during hypoxia and reoxygenation, except CuZnSOD, which dropped to 92 ± 3% after reoxygenation ($P < 0.05$). In cocultivated AC, activities of Cat and GPx did not change during hypoxia, SOD activities decreased slightly (CuZnSOD to 90 ± 6%, MnSOD to 79 ± 10%; $P < 0.05$). After reoxygenation, the activities of Cat, CuZnSOD and MnSOD were diminished to 62 ± 13%, 92 ± 5% and 70 ± 15%, respectively ($P < 0.05$), GPx remained unchanged.

Fig. 2 shows that the content of malondialdehyde increased to 218 ± 82% in EC monocultures, and to 135 ± 10% in AC monocultures after hypoxia and reoxygenation, compared to the respective control ($P < 0.01$ each). If cells were cocultivated, hypoxia/reoxygenation only resulted in a slight, but not significant increase in the content of malondialdehyde in EC (to 121 ± 14%), and AC (to 118 ± 7%), compared with the respective controls. In the case of EC, the cocultivation with AC dramatically reduced the accumulation of malondialdehyde by about 100% ($P < 0.05$). Simultaneously, the survival rate of EC, after hypoxia/reoxygenation, was improved by cocultivation (99% viability), in comparison with that of EC monoculture (79%).

4. Discussion

Comparing EC and AC in monoculture, it may be concluded that AC possess a higher antioxidative potential than EC, due to elevated activities of MnSOD, Cat and GPx, decomposing reactive oxygen species, and lipid peroxidation intermediates. Moreover, no GSSG is detectable in AC, whereas in EC a small amount of GSSG, an indicator of oxidative processes, is found. This shows less oxidative load in AC than in EC, under control conditions.

During hypoxia, AC exhibit less of a decrease in the activity of the defence enzymes than EC. These data are in accordance with other data which show decreased activities of antioxidatively acting enzymes in EC during hypoxia [17,18]. They also show high resistance of AC to hypoxic stress, and only decreased MnSOD in AC after hypoxia [10]. Corresponding to the lower antioxidative defence potential, monocultivated EC exhibit a three times higher enhancement of radical-induced peroxidation products of membrane phospholipids (by 118%) than monocultivated AC (by 35%), upon hypoxia. Therefore, it is assumed that the higher activities of antioxidatively acting enzymes in AC may protect these cells better against hypoxic events than those in EC. In addition, it can be expected that the higher antioxidative potential of AC, covering the cerebral surface of EC in vivo, prevents EC injury by reactive oxygen species when liberated from the brain [5] during pathological situations with oxidative stress, such as ischemia/reperfusion or inflammation.

Malondialdehyde (MDA) formation is taken as an indicator of radical-induced cell injury in this study. Enhanced generation of MDA during hypoxia and reoxygenation has also been observed in earlier studies of endothelial cells, using the same experimental conditions [12,16]. The MDA accumulation in EC occurs simultaneously with anaerobic glycolysis, energy depression, loss of cytosolic enzymes, cell membrane leakage, membrane disruption, and cell death [12]. During hypoxia/reoxygenation, the MDA production is accompanied by a rise in the content of 4-hydroxynonenal, which is prevented by radical scavengers [16]. 4-Hydroxynonenal represents lipid peroxidation processes only induced by reactive oxygen species (ROS) [19]. Thus, the assumption is supported that ROS are liberated in the EC during hypoxia/reoxygenation. This explains why, after hypoxia, higher lipid peroxidation is found in EC monocultures, containing relatively low activities of ROS-decomposing enzymes, than in AC, containing a significantly higher ROS-decomposing potential. The relevance of ROS and ROS-mediated membrane lipid peroxidation in causing hypoxia-related disturbances in EC is confirmed by pharmacological interventions with Cat and SOD. The administration of SOD prevented both the MDA formation and the paraendothelial permeability increase caused in EC cultures by hypoxia/reoxygenation [6]. Also, the addition of Cat may protect the tightness of a macrovascular endothelial cell monolayer during oxidative stress [20].

Cocultivation of EC and AC enhances the activities of anti-

oxidatively acting enzymes, especially that of MnSOD, in both cell types. The coculture system which is used avoids morphological contacts between EC and AC. Therefore, soluble factors have to mediate the increase in enzyme activities in coculture, compared to the respective monocultures. For instance, growth factors such as vascular endothelial growth factor, acidic and basic fibroblast growth factor (bFGF) improve the resistance to oxidative stress in EC [21]. Cytokines, such as tumor necrosis factor-α (TNFα), or interleukin-1 (IL-1), induce MnSOD specifically among antioxidatively acting enzymes in different cell types [22], and induce protection against cerebral ischemia [23,24]. AC and EC can produce TNFα, IL-1, and bFGF [25], which can be intensified by hypoxic events including ischemia/reperfusion [26]. Thus, it may be assumed that the release of growth hormones and cytokines causes the increase in the antioxidative potential in cocultivated AC and EC. The improvement of the antioxidative enzyme capacity in EC by cocultivation is in accordance with studies of the paraendothelial permeability, a functional measure for the tightness of the blood-brain barrier (BBB) formed by EC. These investigations show that the permeability is reduced (and the tightness improved) in EC after coculture with AC, compared to EC monoculture [27]. The expression of BBB markers, such as γ-glutamyltranspeptidase or alkaline phosphatase, is also intensified in EC cocultivated with AC, compared to EC monocultures [28]. Altogether, it is concluded that AC improve BBB properties and the antioxidative potential of EC. The latter would simultaneously protect structures and functions of EC, and hence of the BBB, against pathological situations with oxidative damage.

As a consequence of the cocultivation, MDA formation and cell death, induced by hypoxia/reoxygenation, are substantially reduced in cocultivated EC, compared to monocultivated EC. That means these EC contain appreciable amounts of antioxidants to protect against oxidative damage, as predicted earlier for the BBB [29]. However, the protective effect of cocultivation is probably not only due to the enhanced activity of defence enzymes in EC, but also to the enhanced defence potential in the AC, decomposing reactive species released from EC. The tolerance of lipid peroxidation and viability during hypoxia is in agreement with functional studies. These show that the paracellular permeability of EC cultivated in contact with AC is enhanced only after 2 h of hypoxia [28]; in contrast, EC, in AC-conditioned medium (no AC-EC contact), show the same permeability increase already after 1 h hypoxia [6]. Similar, AC-dependent differences in the resistance against hypoxia/reoxygenation have been reported by [1], who found a protection of the paracellular tightness of EC, cocultivated with AC, in contrast to EC monocultures.

For the first time our data show a supporting effect of AC on EC, with regard to the antioxidative potential. This is similar to what was reported for AC, which can protect neurons from oxidative stress [11]. AC possess a higher antioxidative potential than EC and, in addition, AC enhance the activities of antioxidatively acting enzymes in EC. Therefore, cocultivation of EC with AC is expected, at least in part, to maintain the tightness of the EC monolayer during oxidative stress, by enhancement of the antioxidative capacity. In summary, AC induce the antioxidative potential in EC, forming the blood-brain barrier, and hence, EC together with AC form a barrier against radicals, which can be generated in both the vascular system [7] and the brain [5]. Thus, the antioxidative potential of EC in interaction with AC can be regarded as a further essential property of the blood-brain barrier, which is induced by AC.

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