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A combined treatment with 1α,25-dihydroxy-vitamin D_3 and 17β-estradiol reduces the expression of heat shock protein-32 (HSP-32) following cerebral cortical ischemia☆

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Abstract

1α,25-$(OH)_2$-vitamin D_3 (1,25-D_3) and 17β-estradiol are both known to act neuroprotectively in certain experimental in vitro and in vivo settings and it has been noted that both steroids lead to an upregulation of certain neurotrophic factors. Here, we studied the effects of 1α,25-$(OH)_2$-vitamin D_3 or 17β-estradiol or their combined application on heat shock protein-32 (HSP-32) distribution after focal cortical ischemia using the well established photothrombosis model. Heat shock protein-32 is a well-established marker of the cerebral oxidative stress response and contributes to neuroprotection by metabolising cytotoxic free heme to carbon monoxide, iron and biliverdin. Photothrombotically lesioned rats were injected i.p. 1 h after injury with either 1 μg 1α,25-$(OH)_2$-vitamin D_3/kg or 7 μg 17β-estradiol/kg or a combination of both steroids. Groups of non-lesioned steroid-treated rats and lesioned, solvent-treated rats served as controls. In contrast to non-lesioned rats, in lesioned animals a significant increase in heat shock protein-32 expression occurred which was slightly, but non-significantly altered in the groups treated either with 1α,25-$(OH)_2$-vitamin D_3 or 17β-estradiol alone when compared to the solvent-treated control group. Only the combined treatment with 1α,25-$(OH)_2$-vitamin D_3 and 17β-estradiol resulted in a significant reduction of glial heat shock protein-32 immunoreactivity within the lesion-remote cortical areas supplied by the affected middle cerebral artery (MCA), indicating that both steroids act synergistically in a protective manner.

Keywords: Vitamin D_3; Estradiol; Heme oxygenase; Brain; Ischemia; Neuroprotection

1. Introduction

For the secosteroid 1α,25-$(OH)_2$-vitamin D3 (1,25-D_3), a multitude of new functions have been recognized including its cerebral localization [1,2] as well as specific actions within the brain itself or within brain derived cultures of various cell types [3,4]. Therefore, 1,25-D_3 seems not only to act at specific cerebral target sites but displays almost characteristic features specified for true neurosteroids. In addition, several authors reported a neuroprotective activity for 1,25-D_3 at the level of cell cultures as well as in in vivo animal models of stroke and multiple sclerosis [3,5,6]. It has been proposed that most of the neuroprotective potential of 1,25-D_3 is related to the upregulation of neurotrophic factors such as NGF, NT-3 and GDNF[3]. Similar neuroprotective mechanisms are known for estradiol (E), which also stimulates the expression of neurotrophic factors including BDNF [7,8].

Therefore, it was hypothesized that the combination of both E and 1,25-D_3 could induce additive neuroprotective effects which would be most beneficial from a therapeutic point of view, because both steroids cross the blood–brain barrier (BBB) freely. Furthermore, for both steroids drugs have been developed which are already approved for clinical use.

Asking the question as to why acute cerebral injury may cause a dysbalance in peripherally synthesized steroid hormones such as 1,25-D_3 and E, which can freely cross the BBB, leads to the work of Gacad and Adams [9], who reported that proteins of the heat shock family-70 (HSP-70)

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specifically bind 25-$(OH)_2$-vitamin D_3 and 17β-E. HSP-70 is a widely studied member of the HSPs, for which it is well established that it becomes rapidly induced as an early consequence of many neuropathological conditions including cerebral ischemia [10]. Furthermore, it may have the potential to affect the concentration of both steroid hormones.

In the photothrombosis model for cerebral cortical ischemia, we had noticed that another heatshock protein, HSP-32 (heme oxygenase-1), becomes rapidly induced in glial cells, were it prevents perilesional iron accumulation and serves as a sensitive marker for cerebral regions affected by delayed secondary injury, edema and oxidative stress [11,12]. HSP-32 acts neuroprotectively by detoxifying free heme and leading together with the enzyme biliverdin reductase to biliverdin and bilirubin (antioxidant) formation. Furthermore, its expression pattern is sensitive to pharmacological interventions [12,13]. Here, we tested how a combined post-lesional treatment with 1,25-D_3 and 17β-E effects the cerebral expression and distribution pattern of HSP-32.

2. Materials and methods

Male Wistar rats (250–300 g) were photothrombotically lesioned as described in detail previously using Begal rose and cold light [12]. Lesioned rats were assigned to different treatment groups ($n = 6$ each). One group was injected with 100 μl/100 g body weight solvent (sterile peanut oil) containing 4.5% (v/v) ethanol. Two separate groups were injected with solvent containing 1 μg 1,25-D_3/kg. Another group was injected with 7 μg 17β-E/kg and two additional independent groups received a combined injection with 1,25-D_3 + E using the same concentrations as given above. One group of sham-lesioned rats was treated with solvent, and 3 groups of normal control rats were injected with 1,25-D_3, E, or 1,25-D_3 + E, respectively. In order to use an experimental setting relevant for stroke therapy rats were not treated before lesioning but received their assigned treatment 1 h after lesion induction. After 48 h, all rats were deeply anesthetized with 50 mg sodium pentobarbital/100 g and perfusion fixed using Zamboni's fixative. All histological and immunohistological procedures for HSP-32 detection were done as previously described in detail [11,12]. All experiments had been approved by our animal welfare legislation. HSP-32 positive glial cells were counted in certain cortical regions specified in Fig. 1d by evaluating five sections of each individual brain and group without knowledge about the treatment. Since neither sham-lesioned animals nor normal treated control animals showed any signs of glial cortical HSP-32 expression they were treated as one group during statistical analysis. The animals of the two groups which received 1,25-D_3 and the ones which had received 1,25-D_3 + E were first evaluated as individual groups. After statistical analysis had shown that no differences were found among the groups which had received the same treatment corresponding groups were combined for further statistical evaluation using ANOVA (repeated measurement). For further details refer to [12].

1,25-D_3 was a gift from Schering AG, Berlin, all other chemicals were from Sigma, Deisenhofen, Germany.

3. Results

In order to describe regional changes in the cortical upregulation of HSP-32 in a comparable manner to previous studies we refer to the designations as described previously [12]. Neither sham-operated nor normal controls which had been treated with solvent containing the administered steroids showed any signs of glial HSP-32 immunoreactivity. Rather, HSP-32 immunoreactivity existed only in those subcortical neurons which were known to express HSP-32 constitutively. In contrast, all photothrombotically lesioned animals showed significant high upregulation of glial HSP-32 within the rim directly bordering the core of the lesion. Also a slight reduction of the lesion diameter, as seen at the pial surface of the cortex, was indicated for the groups treated with E and 1,25-D_3 + E. However, lesion volume could not be determined quantitatively for these brains, because the already necrotic core of the lesion is often partly lost in a non-reproducible manner (Fig. 1a and b) during sectioning of frozen brains for immunohistochemistry.

Within the rim region represented by columns B and C (Fig. 1d) HSP-32 positive glial cells were numerous and equally distributed throughout all cortical layers. No significant differences could be established for the various treatment groups within the rim (Fig. 1f). In the retrosplenial cortex (Fig. 1d, column A), which is not supplied by the affected middle cerebral artery (MCA) but by a branch of the anterior cerebral artery, very little expression was seen in all animals (Fig. 1f). In the three evaluated lateral, lesion-remote cortical areas which are supplied by the affected MCA (Fig. 1d, columns D–F) neither the treatment with 1,25-D_3 nor the treatment with E revealed significant differences for HSP-32 positive glial cells when compared to lesioned, solvent-treated animals (Fig. 1e and f). However, the combined treatment with 1,25-D_3 + E showed significantly lower levels when compared to the groups treated with solvent, 1,25-D_3 or E, respectively. In general, glial HSP-32 expression was much stronger within the supragranular cortical layers of all lesion-remote regions and most of the glial cells were either found in contact with the pial surface or cerebral blood vessels (Fig. 1c).

4. Discussion

Similarly to previous studies unilateral cortical photothrombosis resulted in a strong and significant rise in perilesional HSP-32 immunoreactive glial cells [12] within the regions supplied by the affected MCA. The time schedule

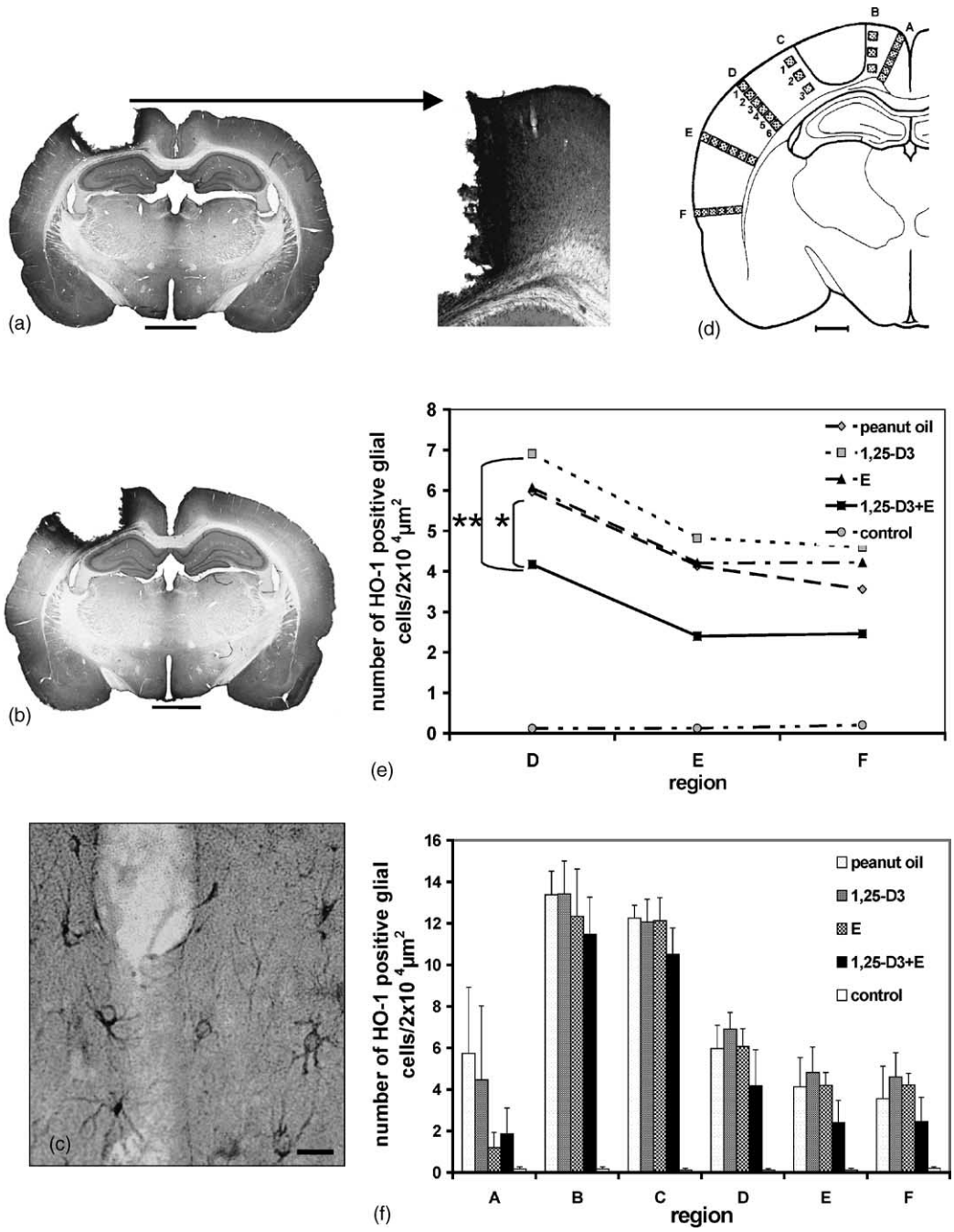

Fig. 1. (a–f) Frontal sections through photothrombotically lesioned rat brains (a: 1,25-D_3-treated; b–c: 1,25-D_3 + E-treated) and the scheme (d) showing the arrangement of the regions (counting fields) depicted for quantitative evaluation of the HSP-32 immunoreactive glial cells as plotted in e and f. The counting fields were arranged in vertical columns to the pial surface to include all cortical layers. HSP-32 immunoreactive glial cells are tightly packed in supragranular and infragranular cortical layers within the rim directly bordering the core (a, inset). In the more remote areas, the packing density of HSP-32 positive glial cells decreases and many of the cells are located in the vicinity of blood vessels (c). In the lesion-remote areas, the number of HSP-32 positive glial cells declines with increasing distance to the core as seen for lateral cortical regions D–F in all animals as seen by the comparable profile of curves (e, for individual standard deviation (S.D.) for each group and region refer to f). Within all three remote regions D–F, the number of HSP-32 immunoreactive glial cells were significantly ($*P \leq 0.05$; $**P \leq 0.001$) lower when compared to solvent-, 1,25-D_3-, or E-treated animals (e and f).

for the lesion-induced HSP-32 expression was similar to previous studies [11] and remained unaffected by the various post-lesional treatments during the first 48 h. Injection of either 1,25-D_3 or E or the combined injection of 1,25-D_3 + E had not caused changes in the cerebral expression or distribution of HSP-32 in non-lesioned control animals, indicating that neither of these hormones affects its expression under normal conditions. The latter suggests that 1,25-D_3 + E may indirectly influences post-lesional HSP-32 expression by altering the expression or actions of

other lesion-induced factors. In the rim region, which is directly associated with the lesion core, the strong HSP-32 upregulation remained unaltered by the various treatments with steroid hormones. This could be due to the rapidly evolving edema within the rim, which may be associated with vascular compression, thus limiting the supply of drugs systemically administered 1 h post-lesion.

However, in the lesion-remote cortical regions usually affected by the secondary, delayed spread of the injury [14], slight, but non-significant changes were found when animals had been treated with 1,25-D_3 or E alone. The finding that only the combined injection of 1,25-D_3 + E caused a significant reduction of HSP-32 immunoreactive glial cells within remote cortical regions indicates that both steroids acted either co-operatively or additive to each other. This action may result in a slow down of the progression of the factors which contribute to the spread of factors contributing to the induction of HSP-32 in regions affected by delayed secondary injury. These factors have to be clearly analyzed in future experiments and may include the 1,25-D_3-induced inhibition of certain calcium channels as well as the 1,25-D_3 or E regulated expression of neurotrophic factors [3,4,6–8]. However, most of the glial cells affected in the lesion-remote cortical areas are associated with blood vessels, thus indicating a direct influence on cells contributing to the formation of the BBB. Since NOS-2 expression and extravasation of systemic heme are well known inducers of HSP-32 [11] it is likely that the previously described 1,25-D_3-induced NOS-2 inhibition [3,15] and the E-induced endothelial NOS-3 expression, which is essential for protecting endothelial cells [16], work hand in hand in stabilizing BBB integrity, thereby preventing the leakage of systemic heme within remote cortical areas supplied by branches of the affected MCA. In conclusion, our results showed a clear cooperative and/or additive action of 1,25-D_3 and E when applied together early after cerebral ischemic insults. The action of both steroids may have a protective potential which needs to be analyzed in detail during future studies.

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