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The antidepressant hyperforin increases the phosphorylation of CREB and the expression of TrkB in a tissue-specific manner

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Abstract

Hyperforin is one of the main bioactive compounds that underlie the antidepressant actions of the medicinal plant \textit{Hypericum perforatum} (St. John’s wort). However, the effects of a chronic hyperforin treatment on brain cells remains to be fully addressed. The following study was undertaken to further advance our understanding of the biological effects of this plant extract on neurons. Special attention was given to its impact on the brain-derived neurotrophic factor (BDNF) receptor TrkB and on adult hippocampal neurogenesis since they appear central to the mechanisms of action of antidepressants. The consequences of a chronic hyperforin treatment were investigated on cortical neurons in culture and on the brain of adult mice treated for 4 wk with a daily injection (i.p.) of hyperforin (4 mg/kg). Its effects on the expression of the cyclic adenosine monophosphate response element-binding protein (CREB), phospho-CREB (p-CREB), TrkB and phospho-TrkB (p-TrkB) were analysed by Western blot experiments and its impact on adult hippocampal neurogenesis was also investigated. Hyperforin stimulated the expression of TRPC6 channels and TrkB via SKF-96365-sensitive channels controlling a downstream signalling cascade involving Ca\textsuperscript{2+}, protein kinase A, CREB and p-CREB. \textit{In vivo}, hyperforin augmented the expression of TrkB in the cortex but not in the hippocampus where hippocampal neurogenesis remained unchanged. In conclusion, this plant extract acts on the cortical BDNF/TrkB pathway leaving adult hippocampal neurogenesis unaffected. This study provides new insights on the neuronal responses controlled by hyperforin. We propose that the cortex is an important brain structure targeted by hyperforin.

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Key words: Antidepressants, CREB, neurogenesis, PKA, TrkB, TRPC6.

Introduction

The medicinal plant \textit{Hypericum perforatum}, also named St. John’s wort (SJW), contains nearly two dozen of bioactive compounds which possess interesting pharmacological properties including: anti-bacterial, anti-inflammatory, anti-tumoural and anti-angiogenic properties (Lorusso \textit{et al.} 2009; Saddique \textit{et al.} 2010; Schempp \textit{et al.} 1999). In addition, SJW extracts have been shown to possess central nervous system (CNS) effects and are used as antidepressants (Di Carlo \textit{et al.} 2001; Linde \textit{et al.} 1996; Mennini & Gobbi, 2004; Muller, 2003). Although several constituents of SJW seem to contribute to its therapeutic efficacy (Chatterjee \textit{et al.} 1998\textit{b}), the phloroglucinol derivative hyperforin is regarded as the main effector (Cervo \textit{et al.} 2002; Chatterjee \textit{et al.} 1998\textit{a}; Zanoli, 2004). However, its actions on brain cells and the mechanisms
underlying its putative clinical antidepressant effect remain poorly characterized. Recently, many cellular targets of hyperforin have been identified (Beerhues, 2006; Medina et al. 2006). For instance, it has been determined that this plant extract controls the activity of enzymes like microsomal prostaglandin E2 synthase-1 (Koeberle et al. 2011), sirtuins 1 and 2 (Gey et al. 2007), cyclooxygenase-1 or 5-lipoxygenase (Albert et al. 2002), as well as binding to intracellular receptors like the pregnane X receptor (Moore et al. 2000) and altering the expression of genes, e.g. CYP2C genes (Chen & Goldstein, 2009). Hyperforin has also been shown to interfere with neurotransmission by inhibiting the re-uptake of many neurotransmitters and dissipating the pH gradient of synaptic vesicles. This results in a redistribution of monoamines from these vesicles to the cytoplasmic compartment (Roz & Rehavi, 2004). Another interesting characteristic of hyperforin is its ability to influence the transport of ions. Hyperforin is a potent blocker of various channels like ionotropic GABA and glutamate receptors (Chatterjee et al. 1999; Fisunov et al. 2000; Kumar et al. 2006). Besides these inhibitory properties, hyperforin is currently known to activate TRPC6, a Ca\(^{2+}\) -conducting channel of the plasma membrane (Leuner et al. 2007). TRPC6 is the only channel opened by this plant extract. The hyperforin-dependent activation of TRPC6 channels controls various biological processes. For instance, when chronically applied, hyperforin promotes neurite extension in the neuronal cell line PC12 (Leuner et al. 2010) and induces the differentiation of keratinocytes (Muller et al. 2008).

The aim of the present study was to advance our understanding of hyperforin’s mechanisms of action on brain cells. In particular, special attention was given to its impact on the brain-derived neurotrophic factor (BDNF) receptor TrkB and on adult hippocampal neurogenesis since they appear central in the molecular pathophysiology of depression and in the pharmacological mechanisms of action of antidepressants (Duman & Monteggia, 2006; Kozisek et al. 2008; Miguel-Hidalgo & Rajkowska, 2002; Sahay & Hen, 2007). Thus, the question of whether hyperforin affects the above-mentioned processes was addressed by using neurons in culture and adult mice chronically treated with hyperforin. The data collected illustrate that chronic hyperforin treatment triggers an intracellular signalling pathway that involves the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and the transcription factor cAMP response element-binding protein (CREB), and regulates the expression of the BDNF receptor TrkB and TRPC6. This hyperforin-dependent cascade critically depends on the activity of Ca\(^{2+}\) -conducting channels and occurs specifically in the cortex but not in the hippocampus. Our findings provide new insights into the neurobiological actions of a chronic hyperforin treatment on CNS neurons.

**Methods**

**Cell cultures**

Primary cultures of cortical neurons were prepared from embryonic E13 C57BL6/J mice (Bouron et al. 2005). Brains were placed in an ice-cold Ca\(^{2+}\) - and Mg\(^{2+}\) -free Hank’s solution to which was added 33 mM glucose, 4.2 mM NaHCO\(_3\), 10 mM Hepes, and 1% penicillin/streptomycin. Depending on the number of embryos, 1–3 pregnant mice were used to prepare one batch of cultured cells. The cortices were pooled and placed in a sterile 1.5 ml Eppendorf tube before being dissociated by means of repetitive aspirations through a sterile and fire-polished Pasteur pipette. This cell suspension was then filtered through a 40 \(\mu\)m cell strainer (BD Falcon, USA) and plated onto poly-L-ornithine-treated Petri dishes (Ø 6 cm). Cells were grown for 3 d in a Neurobasal medium containing 2% B27, 1% penicillin/streptomycin and 500 \(\mu\)M glutamine (Bouron et al. 2005). Next, half of this culture medium was removed and replaced with fresh Neurobasal medium. The procedures used were approved by the Ethical Committee of Grenoble (ComEth) (protocol 7 _IRTSV-LCBM-AB-01).

**Chronic treatment of C56Bl6/J mice with hyperforin**

Twenty-six 5-month-old male C57Bl6/J mice (Charles River, France) were housed under standard conditions on a 12-h light/dark cycle (lights on 07:00 hours) with water and food available _ad libitum_. Thirteen animals received a daily intraperitoneal (i.p.) injection of a fresh hyperforin solution (4 mg of hyperforin/kg body weight) diluted in a sterile NaCl (0.9%) solution. Control animals (\(n = 13\)) were treated with a hyperforin-free NaCl solution. After 4 wk of treatment, the brain of three hyperforin-treated mice and of three control mice were isolated and placed in an ice-cold saline solution. They were subsequently used for the extraction of proteins (see below). The other animals received two 5-bromo-2-deoxyuridine (BrdU) injections at 24-h intervals (25 mg BrdU/animal). After this, the daily injections of hyperforin (or NaCl) were repeated for 3 more weeks before intra-cardiac injection of paraformaldehyde (PFA) and processing of the brains for analysis of hippocampal neurogenesis (see below).
**Protein extraction and Western blot analysis**

For the *in-vivo* experiments, proteins from the cortex and the hippocampus were extracted using Nucleospin RNA/protein kit (Macherey-Nagel, France) followed by precipitation with ethanol. Samples were kept on ice for 30 min and centrifuged at 11,320×g for 30 min at 4°C. The pellet was resuspended in a buffer containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.24% Tris–HCl and 0.5% deoxycholate.

For the *in-vitro* experiments, hyperforin was added to the culture medium 5 d after the plating of the neuronal cells. At that time, the culture medium was renewed and replaced by a fresh Neurobasal medium containing 1 μM hyperforin (or DMSO, control cells). The concentration of DMSO was <0.1%. Cells were subsequently kept for 72 h during which the culture medium containing hyperforin (or DMSO) was changed every day. At the end of the 72-h treatment, cells were harvested and centrifuged for 5 min at 453×g. The pellet was resuspended in a buffer containing (in mM): 10 Hepes, 3 MgCl₂, 40 KCl, 2% glycerol, 1% Triton X-100, 1% protease inhibitors (Sigma, France), with a pH of 7.5. The samples were kept on ice for 30 min and centrifuged at 11,320×g for 30 min at 4°C before collecting the supernatant. Proteins were quantified using the Bio-Rad DC protein assay.

Immunoblotting analyses were performed with 50 μg proteins after separation on a 8% SDS-polyacrylamide gel. PVDF membranes were blocked for 2 h in 5% non-fat dry milk. The primary antibodies used were either anti-TrkB (1:500, Millipore, USA), anti-phospho-TrkB (anti-p-TrkB) (1:200, tebu-bio, France), anti-CREB (1:1000, Millipore, USA), anti-phospho-CREB (anti-p-CREB) (1:1000, Millipore, USA), anti-TRPC6 (1:200, Alomone Labs) or actin (1:250, Sigma). Overnight incubation at 4°C with the primary antibody was followed by incubation with a horseradish peroxidase-coupled secondary antibody (1:5000, Bethyl, USA). Detection of the bands by chemiluminescence (ECL, Pierce, USA) was achieved using a Fusion Fx7 apparatus (Wilbert Lourmat, France). Quantification of protein expression was made with ImageJ software: for a given protein, its expression was normalized to the level of actin expression.

**Adult hippocampal neurogenesis**

For these experiments, 10 animals from each group (i.e., hyperforin-treated and NaCl-treated animals) were used. Animals were anaesthetized with pentobarbital. The fixation of the brain was achieved after an intra-cardiac injection of 4% PFA in PBS (Dupret et al. 2008). Free-floating sections (40 μm) were processed in a standard immunohistochemical procedure in order to visualize BrdU (1:2000, Accurate Scientific, USA), phosphorylated histone H3 (pH 3, 1:2000, Upstate, USA) and doublecortin (DCX, 1:1000, Santa Cruz Biotechnology, USA), on alternate one-inch sections. The numbers of immunoreactive (IR) cells throughout the entire granular layer of the supragranular and infragranular blades of the dentate gyrus (both sides for BrdU and HH3, and the left side for DCX) were estimated using the optical fractionator method (Dupret et al. 2007).

**Statistical analysis**

Throughout this study, data are presented as means±S.E.M. The statistical significance of the difference between the groups was determined using SigmaStat (version 3.5, Systat Software, USA).

**Materials**

Tissue culture media were obtained from Invitrogen (France). BrdU was obtained from Serva (France). All other reagents were from Sigma-Aldrich (France). Hyperforin was a kind gift from Dr Willmar Schwabe GmbH & Co. (Germany). The extract was a mixture of hyperforin with its homologue adhyperforin (ratio 8:2), prepared as sodium salts (Tu et al. 2009).

**Results**

The consequences of a chronic hyperforin treatment on cell proliferation, cell survival and adult hippocampal neurogenesis were analysed in adult mice after 4 wk of daily i.p. injections (4 mg/kg) of hyperforin. The mice then received two injections of BrdU to label dividing cells followed by a continuous treatment of hyperforin for 3 more weeks. The chronic hyperforin treatment had no effect on cell proliferation as measured by the number of phosphorylated histone H3 by means of an anti-phospho-histone H3 that recognizes histone H3 when phosphorylated at Ser10 (pH 3, Fig. 1a). The survival of 3-wk-old BrdU-labelled cells was also not modified (Fig. 1b). As a consequence, neurogenesis, evaluated by the number of DCX, remained unchanged (Fig. 1c). Thus, under the experimental conditions used, hyperforin did not influence hippocampal adult neurogenesis.

The effects of hyperforin on expression of TrkB in the cortex and hippocampus were also addressed. Adult mice were chronically treated for 4 wk (4 mg/kg.d) with hyperforin. This treatment enhanced the expression of TrkB and p-TrkB in the cortex by nearly 124% and 200% (p<0.05), respectively (Fig. 2a, b).
The expression of CREB and p-CREB, although slightly augmented, was nevertheless not different when compared to their respective controls (NaCl-treated animals) \((p>0.05)\). In marked contrast to the cortex, the expression of the above-mentioned proteins in the hippocampus was not modulated by hyperforin (Fig. 2). This indicates that hyperforin exerts tissue-specific responses.

Since hyperforin influences the expression of TrkB in the cortex but not in the hippocampus, cultures of cortical neurons were prepared to uncover the mechanisms by which this plant extract alters the BDNF pathway. We first determined whether a chronic application of hyperforin changes the expression of CREB, p-CREB, TrkB and p-TrkB (Fig. 3a). A 72 h hyperforin treatment enhanced the expression of CREB, p-CREB and TrkB in cultured cortical neurons by 70\%, 109\% and 71\%, respectively \((p<0.05)\) (Fig. 1b). However, p-TrkB remained unaffected.

It was recently shown that a chronic hyperforin treatment in adult mice influences Zn\(^{2+}\) storage capacities of brain cells by stimulating the expression of metallothioneins (Gibon et al. 2011). Since Zn\(^{2+}\) interferes with the activity of the BDNF/TrkB pathway by activating TrkB independent of its ligand (Huang et al. 2008), we investigated whether the Zn\(^{2+}\) chelator TPEN could prevent the hyperforin-dependent regulation of p-CREB and TrkB. Cortical neurons in culture were kept for 72 h with a medium supplemented with either 1 \(\mu\)M TPEN or 1 \(\mu\)M TPEN + 1 \(\mu\)M hyperforin. With TPEN, hyperforin was still able to enhance the phosphorylation of CREB (Fig. 3c) showing that Zn\(^{2+}\) does not play a role in regulating the hyperforin-dependent cascade leading to increases in the expression of CREB and its phosphorylated form. However, in the presence of TPEN, hyperforin failed to up-regulate TrkB expression (Fig. 3d) suggesting that this metal controls a step in between the transcription factor p-CREB and the expression of TrkB.

Since hyperforin selectively enhances the activity of the Ca\(^{2+}\)-conducting TRPC6 channels, while leaving the other TRPC channels unaffected (Leuner et al. 2007), it is postulated that hyperforin could influence neuronal properties by regulating the cytosolic...
Hyperforin activates the expression of TrkB

Fig. 2. Hyperforin regulates the expression of TrkB in the cortex but not in the hippocampus of adult mice. (a) Representative Western blots showing the expression of TrkB, p-TrkB, CREB and p-CREB in control animals (NaCl, n = 3) and in animals treated for 4 wk with hyperforin (i.p., 4 mg/kg.d, n = 3). (b) The panel shows the changes in the expression level of TrkB, p-TrkB, CREB and p-CREB in both groups in the cortex and (black bar) and the hippocampus (white bar). *p < 0.05 (Kruskal–Wallis one-way analysis of variance followed by a Holm–Sidak test).

Concentration of Ca\(^{2+}\). Experiments were performed to verify whether Ca\(^{2+}\) channels were implicated in the hyperforin-dependent regulation of TrkB expression. Cortical neurons were kept in a medium containing the Ca\(^{2+}\) channel blocker SKF-96365 (5 μM) alone or SKF-96365 (5 μM) + hyperforin (1 μM). In the latter case, SKF-96365 was added 10 min before the addition of hyperforin. In the presence of SKF-96365, hyperforin did not enhance the phosphorylation of CREB (Fig. 3c). It also failed to up-regulate the expression of TrkB (Fig. 3d). The Ca\(^{2+}\) chelator BAPTA-AM was used to determine whether Ca\(^{2+}\) controls the phosphorylation of CREB and TrkB expression. Cortical neurons were thus grown in a medium containing 100 nM BAPTA-AM. Preliminary experiments showed that depending on the concentration used (e.g. at ≥1 μM), a sustained application of BAPTA-AM resulted in cell loss (not shown). Interestingly, low concentrations of BAPTA-AM were sufficient to disturb the hyperforin-dependent expression of p-CREB and TrkB (Fig. 3c,d). These results obtained with SKF-96365 and BAPTA suggest that Ca\(^{2+}\) is a central component, playing a role downstream of hyperforin and upstream of p-CREB and TrkB. Furthermore, cAMP-dependent PKA is known to control CREB activity (Kingsbury et al. 2003; Lonze & Ginty, 2002). Thus, its involvement in the hyperforin-dependent phosphorylation of CREB was investigated. Cells were incubated with hyperforin (1 μM) or with hyperforin+RpAMP (20 μM), a competitive inhibitor of PKA (Murray, 2008). Under the latter condition, the expression of TrkB and p-CREB was not up-regulated (Fig. 3c,d). Instead, the expression of TrkB and p-CREB was reduced by 9% and 34%, respectively. The data summarized in Fig. 3c,d suggest that SKF-96365-sensitive channels, Ca\(^{2+}\) ions and PKA are potentially crucial candidates participating in the hyperforin-dependent regulation of TrkB expression.

The results obtained from the SKF-96365 and BAPTA experiments suggest that hyperforin acts on Ca\(^{2+}\)-conducting channels. TRPC6 channels are the sole Ca\(^{2+}\) channels activated by hyperforin (Leuner et al. 2007). It is important to note that the expression of hippocampal TRPC6 channels increases from postnatal day 0 to postnatal day 14 and then strongly declines, with a weak expression observed during adulthood (Tai et al. 2008). This developmental regulation in the expression pattern of hippocampal TRPC6 channels could explain, at least partially, the lack of responsiveness of this brain structure to hyperforin. The expression of TRPC6 channels in the adult mouse brain was investigated by Western blotting (Fig. 4a). In the cortex of control animals, the level of TRPC6 expression was around four times higher than that observed in the hippocampus (Fig. 4b). In addition, hyperforin treatment increased the expression of TRPC6 in the cortex (p < 0.05) (Fig. 4b) but not in the hippocampus (p > 0.05) (Mann–Whitney) (Fig. 4b).

Discussion

SJW is one of the most commonly used medicinal plant (Wills et al. 2000). Since the first meta-analysis of Linde et al. (1996) showing that extracts of SJW were as effective as standard antidepressants for the treatment of some forms of mood disorders (Linde et al. 1996), the usefulness of this medicinal plant has been documented (Di Carlo et al. 2001; Stevinson & Ernst, 1999). Although several constituents of SJW seem to contribute to its antidepressive action, the phloroglucinol derivative hyperforin is considered as its main effector (Cervo et al. 2002; Chatterjee et al. 1998a). Due to its recognized clinical efficacy, SJW is regarded as an...
alternative treatment for depression. However, the mechanisms by which it acts on neuronal functions and those underlying its behavioural responses are still obscure.

In-vitro and in-vivo experiments illustrate the positive influence of a chronic hyperforin treatment on the expression of TrkB. This up-regulation is observed on cultured cortical neurons from embryonic mice (in vitro data) as well as on brain from adult mice (in vivo data). The hyperforin-dependent regulation of the gene coding for this BDNF receptor can be observed in cultured neurons from immature brains and in vivo on mature brains. The hyperforin-dependent expression of TrkB is abolished in the presence of the Ca^{2+} channel blocker SKF-96365, is sensitive to the Ca^{2+} chelator BAPTA, critically depends on the activity of the serine-threonine PKA and involves the inducible transcription factor CREB. Upon binding to gene promoters p-CREB, the active form of CREB, controls the expression of various proteins like TrkB (Lonze & Ginty, 2002). The action of this transcription factor involves a transcriptional co-activator, the CREB-binding protein (CBP). It is a Zn^{2+}-binding protein participating in the regulation of transcription (Legge et al. 2004; Newton et al. 2000). TPEN may block the expression of TrkB by altering the functions and/or the folding of this Zn^{2+}-binding transcriptional co-activator. Figure 5 is a schematic representation...
showing this hyperforin-dependent signalling pathway. Some differences can be noted when comparing the in-vivo and the in-vitro data. For instance, hyperforin strongly augments the expression of p-TrkB in vivo whereas no change was observed in vitro. These differences could be due to the different duration of the treatment (4 wk in vivo vs. 72 h in vitro) and to the concentrations of hyperforin used. But in both models (adult mice chronically treated and cortical neurons in cultures), the plant extract potently enhances the expression of TrkB.

The findings of the present report show that hyperforin controls a SKF-96365-sensitive signalling cascade, involving PKA and CREB. TRPC6 being the only plasma membrane channels known to date to be activated by this agent, it is proposed that they mediate the hyperforin response. Ca^{2+} ions entering via hyperforin-sensitive TRPC6 channels seem to control, in a PKA-dependent manner, the phosphorylation of CREB and the downstream expression of TrkB.

In the brain of adult mice, TRPC6 channels are present in the cortex but are, however, found at a much weaker level in the hippocampus where their expression is developmentally regulated (Tai et al. 2008). Indeed, TRPC6 expression increases from P0 to P14 and then strongly decreases (Tai et al. 2008). Of note, in the hippocampus of young mice, TRPC6 controls the neuritic outgrowth of immature hippocampal neurons and the formation of excitatory synapses (Tai et al. 2008; Zhou et al. 2008). Therefore, the entry of Ca^{2+} through TRPC6 is able to control downstream cellular responses. Our data support this view and are perfectly in line with reports showing that hyperforin is an activator of TRPC6 channels (Leuner et al. 2007; Muller et al. 2008). In addition, a chronic hyperforin treatment enhanced their expression further illustrating that TRPC6 is a target of this plant extract.
Although, a great number of studies have contributed to a better understanding of the molecular biology of depressive disorder, little is known regarding the precise mechanism of action of antidepressant drugs. According to some authors, the neurotrophin BDNF and the action of antidepressants on the hippocampal adult neurogenesis are two central features involving TrkB receptors. Indeed, some experimental data support the existence of a functional link between BDNF, TrkB, the adult hippocampal neurogenesis and the behavioural effects of antidepressant drugs (Li et al. 2008). According to this model, TrkB has a central role in the pathophysiology of depressive disorders. Of interest, hyperforin augments its expression. However, there is no unequivocal demonstration showing that the adult hippocampal neurogenesis is really necessary to produce the behavioural effects of antidepressants (Sahay et al. 2011). Although the BDNF–neurogenesis model has been well documented, it is debated (DeCarolis & Eisch, 2010; Sapolsky, 2004), notably because it seems to suffer some exceptions (Bessa et al. 2009; David et al. 2009; Surget et al. 2008) suggesting that it may not fully cover the neurobiology of all the antidepressants. First, a decrease in hippocampal neurogenesis does not induce a depressive-like behaviour, indicating that this process is not involved in the pathogenesis of depression (Revest et al. 2009; Santarelli et al. 2003). Importantly, the effects of antidepressants on neurogenesis seem to depend on the mouse strain used (Holick et al. 2008). In addition, antidepressants like fluoxetine seem to stimulate the hippocampal neurogenesis of mice under chronic stress but not when tested on unstressed animals (Surget et al. 2010). Another important parameter to consider when trying to understand the requirement (or not) of hippocampal neurogenesis on the efficacy of antidepressant drugs, is the type of antidepressant used. For instance, hippocampal neurogenesis underlines the action of monoanergic compounds but does not seem to be required for drugs acting on the hypothalamo-pituitary-adrenal (HPA) axis (Surget et al. 2008).

In conclusion, in-vitro and in-vivo experiments were conducted to better address the CNS effects of the plant extract hyperforin. The data presented show that it controls a signalling cascade involving Ca$^{2+}$-conducting channels, PKA and CREB, which, in turn, up-regulates the expression of the BDNF receptor TrkB. Additionally, these responses are observed in the cortex but not in the hippocampus of adult mice where the expression of TRPC6 channels is weak. Altogether, the cortex appears as an important brain structure participating in the CNS effects of hyperforin.

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Statement of Interest

None.

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