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Truffles contain endocannabinoid metabolic enzymes and anandamide

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ABSTRACT

Truffles are the fruiting body of fungi, members of the Ascomycota phylum endowed with major gastronomic and commercial value. The development and maturation of their reproductive structure are dependent on melanin synthesis. Since anandamide, a prominent member of the endocannabinoid system (ECS), is responsible for melanin synthesis in normal human epidermal melanocytes, we thought that ECS might be present also in truffles. Here, we show the expression, at the transcriptional and translational levels, of most ECS components in the black truffle *Tuber melanosporum* Vittad. at maturation stage VI. Indeed, by means of molecular biology and immunochemical techniques, we found that truffles contain the major metabolic enzymes of the ECS, while they do not express the most relevant endocannabinoid-binding receptors. In addition, we measured anandamide content in truffles, at different maturation stages (from III to VI), through liquid chromatography–mass spectrometric analysis, whereas the other relevant endocannabinoid 2-arachidonoylglycerol was below the detection limit.

Overall, our unprecedented results suggest that anandamide and ECS metabolic enzymes have evolved earlier than endocannabinoid-binding receptors, and that anandamide might be an ancient attractant to truffle eaters, that are well-equipped with endocannabinoid-binding receptors.

1. Introduction

Truffles are the fungal subterranean fruiting bodies of the genus *Tuber* (Ascomycota) which depends on animal feeding for spores dispersal. Some of these mushrooms are endowed with major gastronomic and commercial value. The winter black truffle (*Tuber melanosporum* Vittad.) is the most renowned species, and for this reason it has been extensively studied and its genome has been fully sequenced (Martin et al., 2010). Analysis of the biochemical composition of truffles has shown that rhamnose, calcium, iron and a relatively high level of melanin (~15% by dry weight) are typical components, that could be also used as biomarkers of the degree of ascocarp development and of the attainment of maturity (Harki et al., 1997). In addition, truffle sexual reproduction and fruit body development are dependent on melanin synthesis (Heung et al., 2005; Engh et al., 2007), as in the case of many ascomycetes and basidiomycetes (e.g., *Neurospora crassa*, *Coenococcum*, *Cryptococcus*, *Podospora anserina*, *Sordaria macrospora*, *Schizophyllum commune*, *Agaricus bisporus*, *Morchella*, *Ophiostoma piliferum*) (Hirsh, 1954; Esser, 1968; Horowitz et al., 1970; Prade et al., 1984; Zimmerman et al., 1995; Miranda et al., 1997; Heung et al., 2005; Engh et al., 2007). Indeed, mRNA expression of tyrosinase, the key-enzyme in this process (Miranda et al., 1997; Gerdermann et al., 2002; Simon et al., 2009), increases during truffle development (Pacioni et al., 1995), and decreases in relation to thioflavour production (Zarivi et al., 2011). The roles of fungal melanins arising from c-glutaminyl-3,4-hydroxybenzene (GHB) and 1,8-dihydroxynaphthalene (1,8-DHN) oxidations (allomelanins),

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as well as those of eumelanins (nitrogen-containing melanins) and phaeomelanins (sulfur- and nitrogen-containing melanins) are pleiotropic. In white (*Tuber magnatum*, *Tuber borchii* Vittad.) and black (*T. melanosporum*, *Tuber aestivum* Vittad., *Tuber brumale* Vittad.) truffles the expression of tyrosinase and melanin synthesis are cogently related to the reproductive differentiation (Miranda et al., 1996, 1997). *T. melanosporum* melanin has been partially characterized (De Angelis et al., 1996; Harki et al., 1997, 2006), unlike that of white truffles. It has been suggested that yellowish melanin of white truffles may be a kind of thio-melanin (Miranda et al., 1997). In *T. melanosporum* genome three laccase genes have been found, Tmellcc1, Tmellcc2, Tmellcc3 and two tyrosinase genes, Tmeltyr1 and Tmeltyr2, the expressions and enzyme kinetics of which have been characterized. Homology investigations have shown in *T. melanosporum* genome a polyketide synthase (PKS) gene, but not the gene coding for scytalone dehydratase, a key enzyme for melanin synthesis through the DHN pathway (Bell et al., 1976; Nosanchuk and Casadevall, 2003). However, *Aspergillus niger* and basidiomycetes do not have this pathway, thus many ascomycetes and some imperfect fungi appear to make DHN melanin, whereas basidiomycetes and other imperfect fungi use alternative routes (Wheeler, 1983). At present, the biosynthetic pathway of truffle melanin is not quite clear. In this context, we have recently demonstrated that anandamide (*N*-arachidonoylethanolamine, AEA), an endocannabinoid (eCB) that binds to type-1 (CB_1) and type-2 (CB_2) G protein-coupled cannabinoid receptors, dose-dependently stimulates melanin synthesis and enhances tyrosinase gene expression and activity in normal human epidermal melanocytes (Pucci et al., 2012). AEA, CB_1 and CB_2 belong to the endocannabinoid system (ECS), that includes additional eCBs-binding receptors, like the GPR55 (purported "CB_3") receptor (Ross, 2009; Gasperi et al., 2013) and the transient receptor potential vanilloid 1 (TRPV1) channel (Di Marzo and De Petrocellis, 2010). Moreover, ECS contains another bioactive lipid called 2-arachidonoylglycerol (2-AG), and the metabolic enzymes responsible for eCBs synthesis and degradation: *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH), for AEA; diacylglycerol lipase (DAGL) and monoacylglycerol lipase (MAGL), for 2-AG (Maccarrone et al., 2010a; Battista et al., 2012).

The evolution and comparative biology of the ECS has been characterized in mammalian and non-mammalian vertebrates (for a review see Elphick, 2012). AEA and 2-AG metabolic enzymes as well as cannabinoid receptors evolution have been investigated by the search for functional orthologues from phylogenetically different organisms. Such an approach has demonstrated the presence of NAPE-PLD and FAAH, as well as that of DAGL and MAGL in the animal kingdom, in vertebrates and invertebrates, although the loss of NAPE-PLD and MAGL genes has been reported in some lineages, e.g. in *Drosophila* (Elphick, 2012). On the other hand, eCBs-binding receptors have been identified in vertebrates, whereas an orthologue (termed CiCBR) has been found in the deuterostomes uro-chord *Ciona intestinalis* (Elphick et al., 2003). CB_1 and CB_2 seem to be present only in the phylum *Chordata*, thus a limited phylogenetic distribution in the animal kingdom has been reported so far (Elphick, 2012). Instead, orthologues of vertebrate cannabinoid receptors have not been found in fungi and plants (McPartland et al., 2007).

Against this background, here we sought to investigate the presence of ECS in truffles at the full maturation (stage VI), where the highest melanin content is detected and spores are ready to be dispersed by the animal truffle eaters (Pacioni et al., 1995). In addition, we checked whether any relationship may exist between melanin and AEA at different maturation stages (from III to VI) of *T. melanosporum*.

2. Results and discussion

In the first series of experiments, truffles were shown to contain mRNAs of the metabolic enzymes of AEA (NAPE-PLD and FAAH) and of 2-AG (DAGL and MAGL), whereas none of the major eCBs-binding receptors could be detected by qRT-PCR (Fig. 1a). These data were obtained by using primers designed on *T. melanosporum* sequences (Table 1). In addition, since the Blast alignment analysis against the sequences of the main human eCBs-binding receptors (CB_1, CB_2, GPR55 and TRPV1) in the GenBank database of plants (taxid: 3193) and fungi (taxid: 4751) did not show any sequence homology (Supplementary Table S1), a set of primers specific for the mouse genes (Bari et al., 2011) was also tested (Supplementary Table S2). With the mouse primers, no specific amplification products were obtained in truffles (data not shown), further suggesting that these fungi express only eCBs metabolic enzymes. Incidentally, it should be recalled that genes encoding for other G protein-coupled receptors have been previously identified in the truffle genome (Martin et al., 2010). Consistently with the mRNA data, Western blot analysis showed a well-detectable expression of all metabolic enzymes (Fig. 1b). Moreover, densitometric analysis of immunoblots revealed NAPE-PLD to FAAH (=1.49) and DAGL to MAGL (=0.21) ratios, that were indicative of a more efficient synthesis of AEA and degradation of 2-AG (Fig. 1c). In keeping with these data, the endogenous content of AEA was rather high (7.0 ± 5.8 pmol/mg protein), whereas that of 2-AG was below the detection limit of 0.2 pmol/mg protein (Fig. 1d).

Incidentally, AEA content was not detectable at the early stages of maturation (III and IV) of *T. melanosporum*, whereas a significant increase (from 0.54 ± 0.20 to 6.64 ± 1.85 pmol/mg protein, $p < 0.01$) was observed at maturation stages V and VI, respectively (Fig. 2a and b). In addition, taking into account that melanin content increases from stage III to VI (Fig. 2c), these data support a link between AEA content and the melanization process in the developing truffle.

Our data show, for the first time, the presence of ECS components for both AEA and 2-AG metabolism in truffles, along with AEA, but not 2-AG, in the fruit body. On the basis of the observation that truffles have an high NAPE-PLD to FAAH ratio and a low DAGL to MAGL ratio, consistent with a well-detectable content of AEA but not of 2-AG, we can conclude that truffles produce AEA only. Consistently with literature data (McPartland et al., 2006), we found that truffles do not express the major eCBs-binding receptors, suggesting that in these fungi eCBs are unlikely to regulate biological processes like melanin synthesis, maturation and differentiation, that in animals are controlled by eCBs through CB_1/CB_2-dependent mechanisms (Galve-Roperh et al., 2013; Maccarrone, 2013; Pucci et al., 2013; Xapelli et al., 2013). On the other hand, AEA might stimulate the maturation and melanization processes of truffles, since high AEA levels were found in the late stages (V and VI), where also melanin content is high. Yet, the presence of AEA in ripe truffles favors the hypothesis that this eCB might play a role in truffle interaction with the surrounding environment. In this context, it should be recalled that truffle aroma is composed of many volatile compounds of low molecular weight, referred to as VOCs (volatile organic compounds that include a complex mixture of alcohols, aldehydes, aromatic compounds, esters, furans, hydrocarbons, ketones, and nitrogen- and sulfur-containing compounds) (Splivallo et al., 2011). Truffle aroma readily diffuses through the soil, to reach the ground surface where it attracts animal vectors for spore dispersal (Pacioni et al., 1991). Therefore, it can be proposed that the presence of AEA in truffles might represent a nutritional reward to truffle eaters, like long-footed potoroo, meerkat, chacma baboon and grizzly bear (Trappe and Claridge, 2010). Remarkably, these animals are all mammals, that are

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