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TITLE: Anandamide modulates growth and lipid metabolism in the zebrafish *Danio rerio*

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**SUMMARY**

Endocannabinoids are known to be lipidic mediators with several biological functions as the stimulation of food intake and lipid metabolism *via* cannabinoid receptor CB1. Many evidences, such as the presence of *CB1* mRNA in fat tissue, suggest a peripheral role for endocannabinoids in regulating lipogenesis and body weight in mammals.

As animal models constitute good tools to study endocannabinoid system dynamics, we analyzed the role of the endocannabinoid anandamide (AEA) in modulating lipid metabolism and growth in zebrafish larvae and adults.

The data obtained indicated that AEA administered *via* water modulates the transcription of its own receptor *CB1*, besides to up-regulate gene expression of sterol regulatory element binding protein (*SREBP*) and of the insulin-like growth factors (*IGF-1* and *IGF-2*).

The results here obtained represent the first evidence in fish of the endocannabinoid system involvement in lipid metabolism and growth.

**Key words:** endocannabinoids, AM251, *CB1*, *IGFs*, *SREBP*

## INTRODUCTION

During the last few years many different regulatory actions have been attributed to the endocannabinoid system.

Endogenous cannabinoids act at CB1 level stimulating appetite and inducing the increase of body weight (Seeley and Woods, 2003). Maintenance of body weight and energy homeostasis requires the coordinated regulation of appetite behaviour and peripheral energy metabolism as demonstrated by the ability of the endocannabinoid system to interact with leptin, the appetite-reducing hormone, insulin and cholesterol in regulating fat metabolism (Ravinet et al., 2003).

A study conducted on obesity in mice showed that a chronic blockage of CB1 receptor induces a significant reduction in fat mass respect to muscular mass (Ravinet et al., 2003; Poirier et al., 2005), leading to confirm what found by Kunos and collaborators who defined the endocannabinoids as “novel lipid mediators” (2002).

Knock out mice for *CB1* fed on a standard diet since birth, resulted to be leaner respect to wild type fed at the same manner (Ravinet et al., 2004).

Moreover Liu and collaborators (2005) suggested that the anorexigenic effect of AEA antagonist, besides to act on weight and fat mass decrease, also prevents peripheral stimulatory action of endocannabinoids on lipogenesis and fat accumulation.

This hypothesis was supported by the finding of CB1 receptor in mammalian adipocytes whose stimulation brings to lipases activation (Cota et al., 2003), while its blockage brings to the up regulation of adiponectin, a crucial hormone for the inhibition of enzymes involved in lipogenesis (Bensaid et al., 2003; Poirier et al., 2005).

At peripheral level other tissues as liver, pancreas and skeletal muscle are also involved in energy balance cannabinoid system-mediated. In particular, at hepatic level, CB1 receptor stimulation brings to an increase of fatty acid synthesis through the enhancement of *SREBP* and its target genes (acetyl-CoA carboxylase, fatty acid synthase) expression

(Osei-Hyiaman et al., 2005). CB1 activates fatty acid synthase also in the hypothalamus and receptors for the adiponectin were found also in the nucleus paraventricularis demonstrating that the modulation of energy balance is under the endocannabinoid control passing through a cross talk between hypothalamus and peripheral tissues (Qi et al., 2004; Lichtman and Benjamin, 2005).

To date, the role of the endocannabinoid system in lipid metabolism and growth were studied mainly on mammals, especially on rodents. In order to have a greater understanding of the basic and conserved physiological functions controlled by the endocannabinoid system, that shows high similarities among vertebrates (McPartland et al., 2006), zebrafish may represent a good experimental model to study these items, since its full genome availability and its easy breeding and maintenance.

The aim of this paper was, indeed, to explore AEA effects on the expression of *CB1* and genes involved in growth and lipid metabolism in zebrafish larvae and adults. Regarding *CB1* expression, we also tested the effect of one of its antagonist, the AM251

The data obtained also showed, for the first time, the effect of AEA administration *via* water on target genes in whole larvae and adult liver.

## **MATERIALS AND METHODS**

### **Zebrafish exposure to AEA and AM251**

Three groups of 50 larvae each were performed at 72 hours post fertilization (hpf), corresponding to hatching time, and at 7 and 15 days post fertilization (dpf). The first group was used as control, the second was exposed to AEA 10 nM (dose was chosen on the bases of preliminary studies) for two hours and the third to AM251 10 nM for two hours followed by two hours of AEA 10 nM. All the samples were stored at -80°C for the

biomolecular analysis.

Adult zebrafish were instead divided into four experimental groups of 10 fish each: the control group, two groups exposed to AEA, 10 and 100 nM respectively, for two hours and the last group exposed to AM251 10 nM (doses were chosen on the bases of preliminary studies) for the same period. Zebrafish were then anesthetized and the livers were sampled and stored at -80°C until use for biomolecular analysis.

The procedures were performed in accordance with the Guidelines on the handling and Training of Laboratory Animals by the Universities Federation for Animal Welfare (UFAW) and with the Italian animal welfare legislation (D.L. 116/92).

### **RNA extraction and cDNA synthesis**

Total RNA was extracted from 72 hpf, 7 dpf, 15 dpf and liver of adult fish with RNeasy Mini Kit (250) (Quiagen). First strand cDNA synthesis was performed as already described in Migliarini et al. (2006).

### **Real time PCR**

A relative quantification of *CB1*, *IGF-1*, *IGF-2* and *SREBP* gene expression was made by Real time PCR using the acidic ribosomal protein (ARP) as internal standard. In order to amplify these genes, the following primer pairs were designed: *FCB1*: 5'-TCTGTGGGAAGCCTGTTT-3', *RCB1*: 5'-ACCGAGTTGAGCCGTTTG-3', *FIGF-1*: 5'-GGCAAATCTCCACGATCTCTAC-3', *RIGF-1*: 3'-CGGTTTCTCTTGTCTCTCTCAG-5', *FIGF-2*: 5'-GAGTCCCATCCATTCTGTTG-3', *RIGF-2*: 3'-TCCTTTGTTTGTGTCATTG-5', *FSREBP*: 5'-GCTGTTACCCTCTGCTGAAG-3', *RSREBP*: 5'-TGAAACCGCTGCCTTGAC-3'.

*ARP* was amplified using the following primers: *FARP*: 5'-TTCCTCGGTATGGAGTCCT-3', *RARP*: 5'-TGGGGCAATGATCTTGATCTT-3'.

The Real Time PCRs were performed with SYBR green method in a iQ5 Multicolor Real-Time PCR Detection system (BioRad). Triplicate PCR reactions were carried out for each sample analyzed.

The reactions were set on a 96-well plate by mixing, for each sample, 1  $\mu$ l of diluted (1/10) cDNA, 5  $\mu$ l of 2X concentrated SYBR Green PCR Master Mix (BioRad), containing SYBR Green as fluorescent intercalating agent, 0,3  $\mu$ M forward primer and 0,3  $\mu$ M of reverse primer. The thermal profile for all reactions was 3 min 95°C and then 45 cycles of 10 s at 95°C, 20 s at 60 °C and 20 s at 72°C. The fluorescence monitoring occurred at the end of each cycle.

Additional dissociation curve analysis was performed and showed in all cases one single peak.

Data obtained were treated by iQ5 optical system software version 2.0.

## RESULTS

### **CB1 gene expression**

The exposure of zebrafish larvae to AEA significantly up regulated *CB1* gene expression in all the developmental stages analyzed: 72 hpf (hatching time), 7 dpf and 15 dpf (Fig. 1).

Similar results were obtained in the liver of adult zebrafish, after AEA *in vivo* exposure, with the highest dose of the endocannabinoid (Fig. 2).

For what concern *CB1* gene expression, the effect of a CB1 antagonist, AM251, was also evaluated in the three developmental stages here studied, as well as in adult liver. AM251 significantly down regulated *CB1* transcription in larvae and in adult liver (Fig. 1-2).



### ***IGF-1* and *IGF-2* gene expression**

Zebrafish larvae exposed to AEA at 72 hpf (hatching time), 7 dpf and 15 dpf showed a significant increase of *IGF-1* gene expression respect to controls (Fig. 3). As regard *IGF-2*, the same results were evident for 7 and 15 dpf larvae while no significant differences were measurable at hatching time respect to control (Fig. 4). The effect of AEA on zebrafish larvae *IGF-1* and *IGF-2* gene expression was the same detected in adult zebrafish liver. In particular, both doses of AEA were effective on *IGF-1* expression while only the highest dose provoked a significant up regulation on *IGF-2* (Fig. 5).

### ***SREBP* gene expression**

*SREBP* gene expression was up regulated by AEA exposure in zebrafish larvae at 7 dpf and 15 dpf, while no significant changes between larvae exposed to AEA and controls were measurable at 72 hpf (hatching time) (Fig. 6). In the liver of the adult zebrafish, only the exposure to the highest dose of AEA induced a significant increase of *SREBP* gene expression respect to controls (Fig. 7).

## **DISCUSSION**

Animal models are considered from decades ideal tools for elucidating the role of exogenous and endogenous cannabinoids in the control of energy metabolism (Huy et al., 1975; McLaughlin et al., 1979; Guzman et al., 1995; Cota et al., 2003).

Contradictory results were obtained depending on the species and the doses of the cannabinoids administered, but it is currently known that the role of the endocannabinoid in the regulation of food responses and energy metabolism presents a high degree of evolutionary conservation.

A recent study conducted on mice indicated that the endocannabinoid system controls energy homeostasis *via* both central orexigenic and peripheral lipogenic mechanisms.

Moreover, they found that in young *CB1*<sup>-/-</sup> mice, the lean phenotype is predominant caused by decreased caloric intake whereas, in adult *CB1*<sup>-/-</sup> mice, metabolic factors appear to contribute to the lean phenotype (Cota et al., 2003).

In this paper we focused on the endocannabinoid AEA effects, mediated by its receptor CB1, on the peripheral lipogenic mechanisms as well as on growth in adult zebrafish liver and in different zebrafish developmental stages starting from hatching.

We first intended to study AEA ability, administered *via* water, to modulate the gene expression of its own receptor *CB1*, and we found that this endocannabinoid up regulates *CB1* transcription as it is well known for other lipophilic molecules such as steroid hormones (i.e. estrogens, androgens and cortisol) able to modulate their own receptors gene expression (Pottinger 1990; Dai and Burnstein, 1996; Meucci and Arukwe 2006; Mortensen and Arukwe 2007). As there are not previous evidences in literature proving the effects of endocannabinoid administration *via* water, these results provide a very interesting evidence on AEA capability to pass zebrafish larval skin and to target adult liver, modulating the transcription of *CB1* from early life cycle to adult life. In adults, the up regulation of *CB1* mRNA occurs with a 10 fold higher AEA dose, respect to larvae.

*CB1* down regulation, operated by the antagonist AM251, proved that its modulation by AEA occurs *via* the CB1-AEA complex as usually evidenced for many lipophilic signals.

As it is known that in *CB1*<sup>-/-</sup> mice the levels of the lipogenic factor SREBP gene expression are consistently lower respect to *CB1*<sup>+/+</sup> (Osei-Hyaman et al., 2005), we then analyzed its transcription levels modulation by AEA. Besides *CB1* expression increase, our results showed that this endogenous cannabinoid, *via* water, also up regulates *SREBP*.

This result is in agreement with previous data obtained in mammals demonstrating that *SREBP* induction is enhanced by *CB1* up-regulation (Osei-Hyaman et al., 2005). More specifically, we found that in the adult fish liver, only the highest dose of the endocannabinoid resulted in the up regulation of SREBP expression. Regarding the

zebrafish developmental stages, AEA became effective on *SREBP* expression at 7 dpf and 15 dpf. No effect was evident at hatching time when the small larvae were not ready to eat by themselves and the energies were supplied by the yolk sack.

Moreover, we analyzed the expression of two factors belonging to the insulin growth factor system: *IGF-1* and *IGF-2*, known to be secreted by adult liver and to target muscles for cell proliferation (Jones and Clemmons, 1995; Chen et al., 1998). The IGFs are also responsible for embryo and larval growth during development (Carnevali et al., 2005).

In this study, as regards the endocannabinoid system control of growth, the *IGF-1* and *IGF-2* were found to be up regulated by AEA with the exception for hatching stage where this endocannabinoid resulted ineffective.

The increase of *IGFs* gene expression may be due to peroxisome proliferator activated receptors (PPARs)  $\alpha$  and  $\gamma$ , known to be involved in lipid metabolism and cell proliferation and development (Braissant and Wahali, 1997; Fu et al., 2005; Grimaldi, 2007), as *IGF-1* is one of PPARs target genes (Villeneuve, 2004). Interestingly, recent studies also described the induction of *PPAR*  $\alpha$  and  $\gamma$  by AEA (Burstein 2005; Russo et al., 2007). These last results further confirmed that AEA, administered *via* water, modulates the expression of target genes and showed, for the first time, a potential relation between the endocannabinoid system and *IGFs* in fish.

It is thus possible to conclude that the results reported in this study altogether constitute a starting point for better understanding the role of the endocannabinoid system in growth and cellular proliferation, besides to represent a useful contribution in the comprehension of the endocannabinoid involvement in lipid metabolism, so far exclusively studied only on mammals.

**Legends**

**Fig. 1** *CB1* gene expression in zebrafish larvae exposed to AEA or AEA+AM251 at hatching time (A), 7 dpf (B) and 15 dpf (C). Different letters indicate significant differences ( $p < 0,05$ ).

**Fig. 2** *CB1* gene expression in the liver of adult zebrafish exposed to AEA 10 or 100 nM and to AM251. Different letters indicate significant differences ( $p < 0,05$ ).

**Fig. 3** *IGF-1* gene expression in zebrafish larvae exposed to AEA at hatching time (A), 7 dpf (B) and 15 dpf (C). Different letters indicate significant differences ( $p < 0,05$ ).

**Fig. 4** *IGF-2* gene expression in zebrafish larvae exposed to AEA at hatching time (A), 7 dpf (B) and 15 dpf (C). Different letters indicate significant differences ( $p < 0,05$ ).

**Fig. 5** *IGF-1* (A) and *IGF-2* (B) gene expression in the liver of adult zebrafish exposed to AEA 10 or 100 nM. Different letters indicate significant differences ( $p < 0,05$ ).

**Fig. 6** *SREBP* gene expression in zebrafish larvae exposed to AEA at hatching time (A), 7 dpf (B) and 15 dpf (C). Different letters indicate significant differences ( $p < 0,05$ ).

**Fig. 7** *SREBP* gene expression in the liver of adult zebrafish exposed to AEA 10 or 100 nM. Different letters indicate significant differences ( $p < 0,05$ ).

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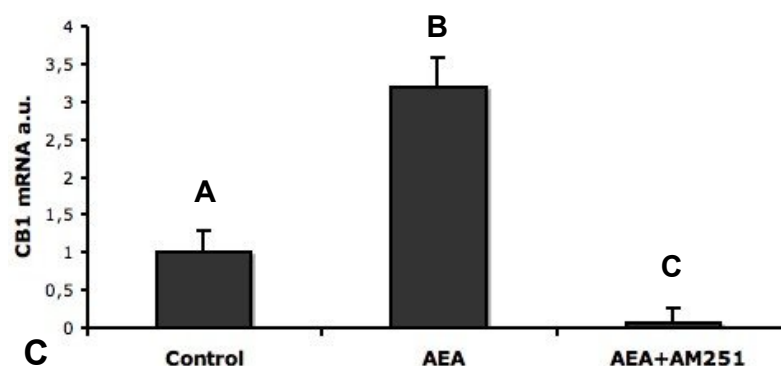
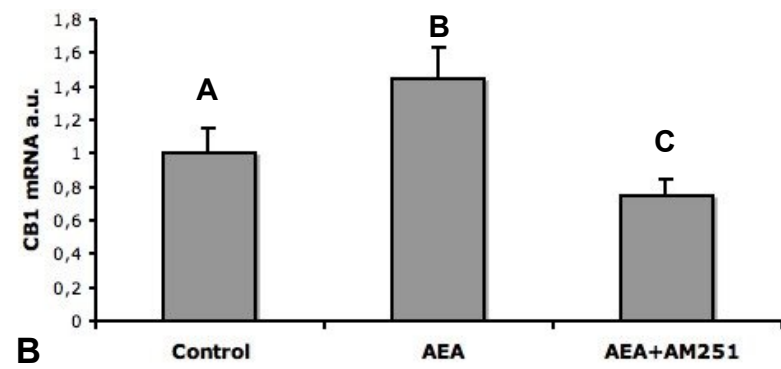
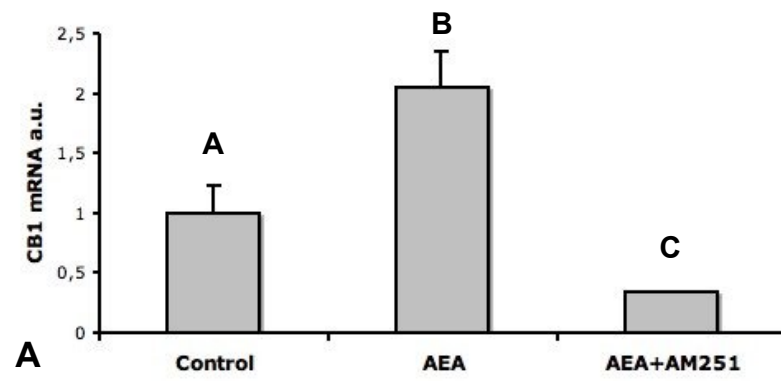
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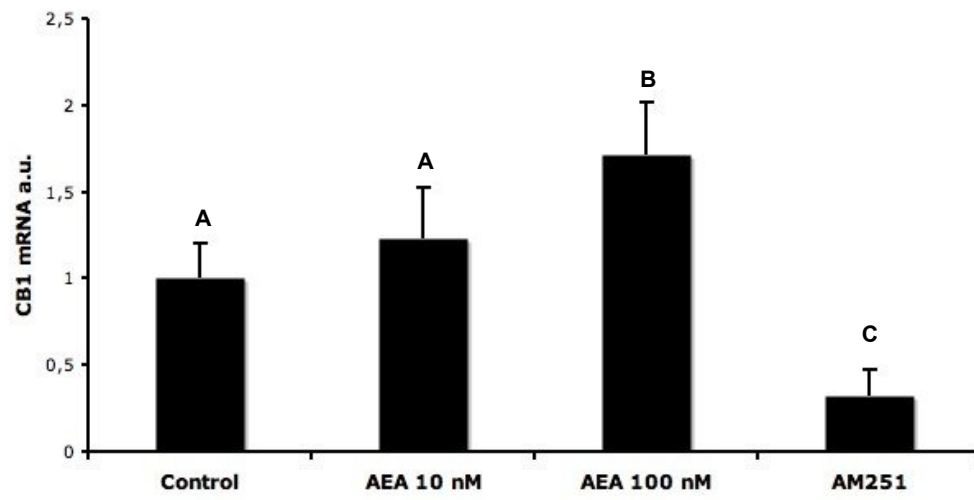
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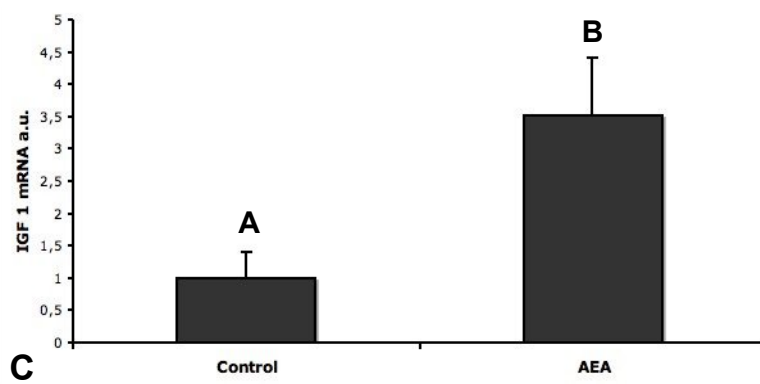
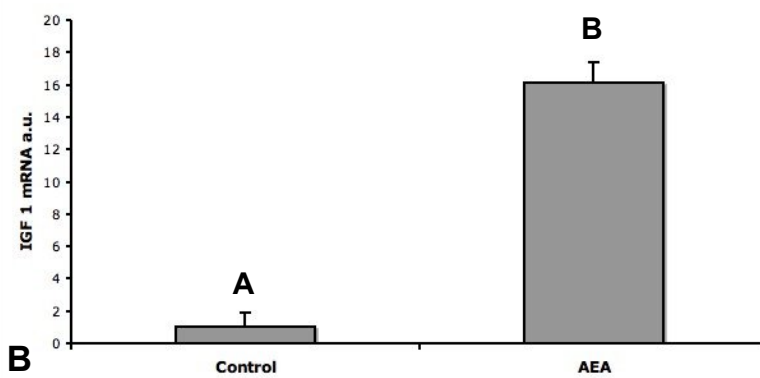
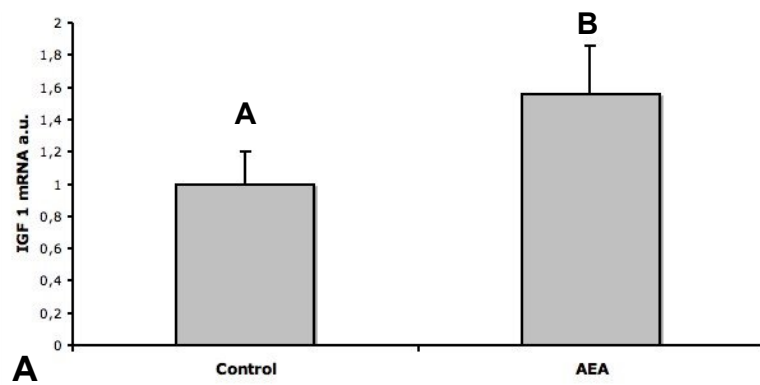
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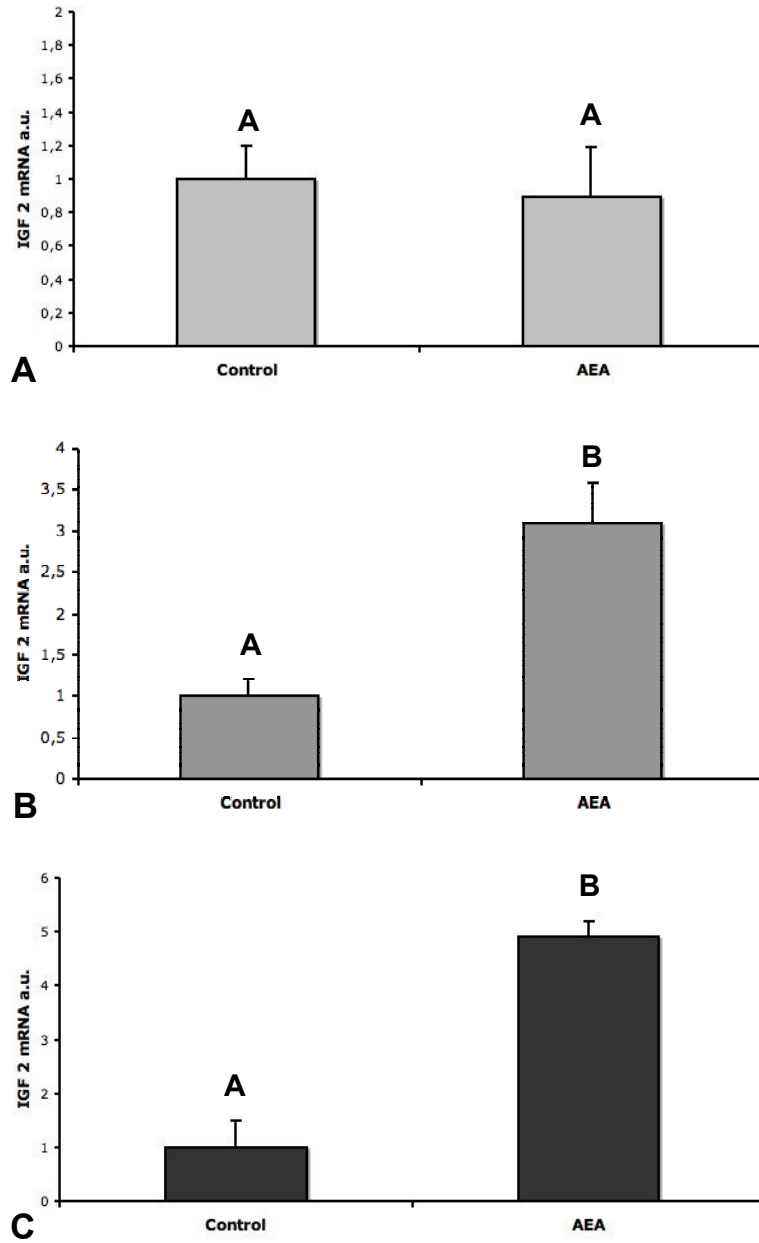


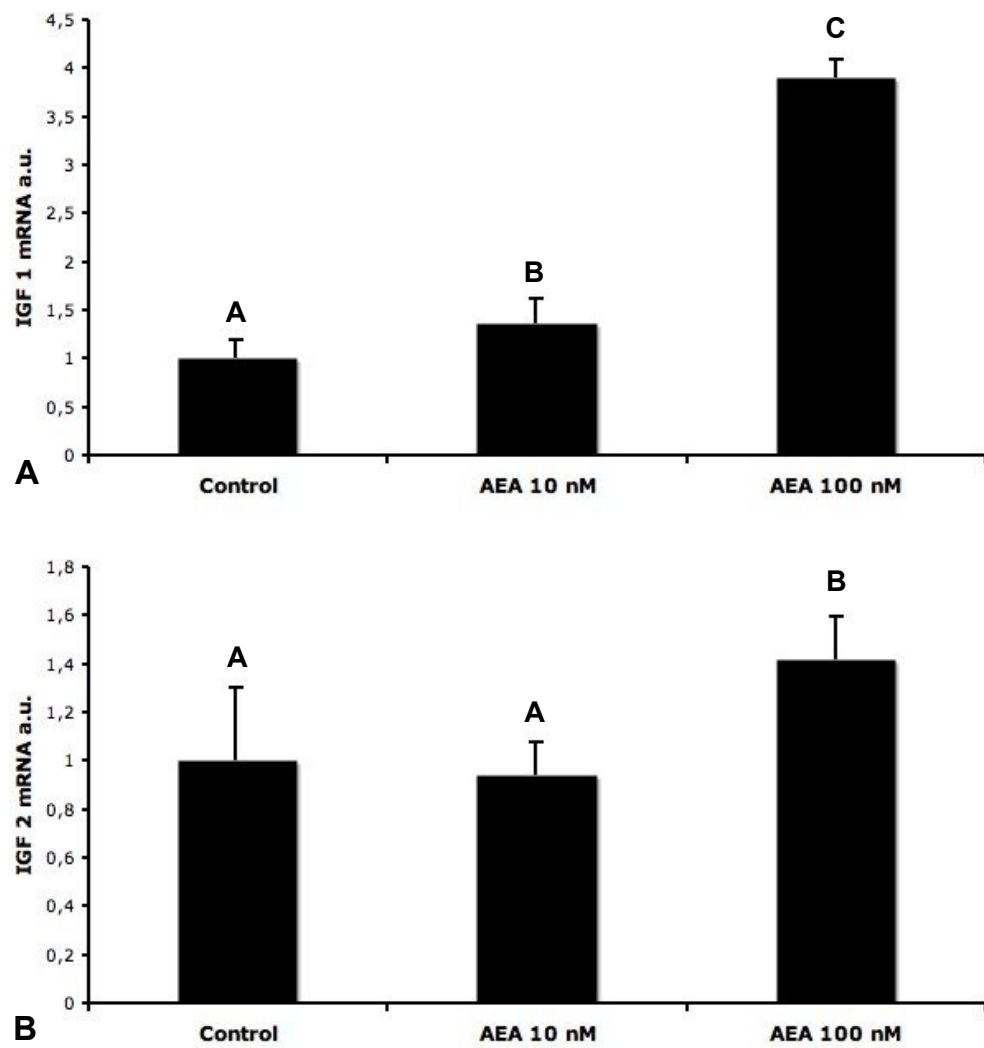


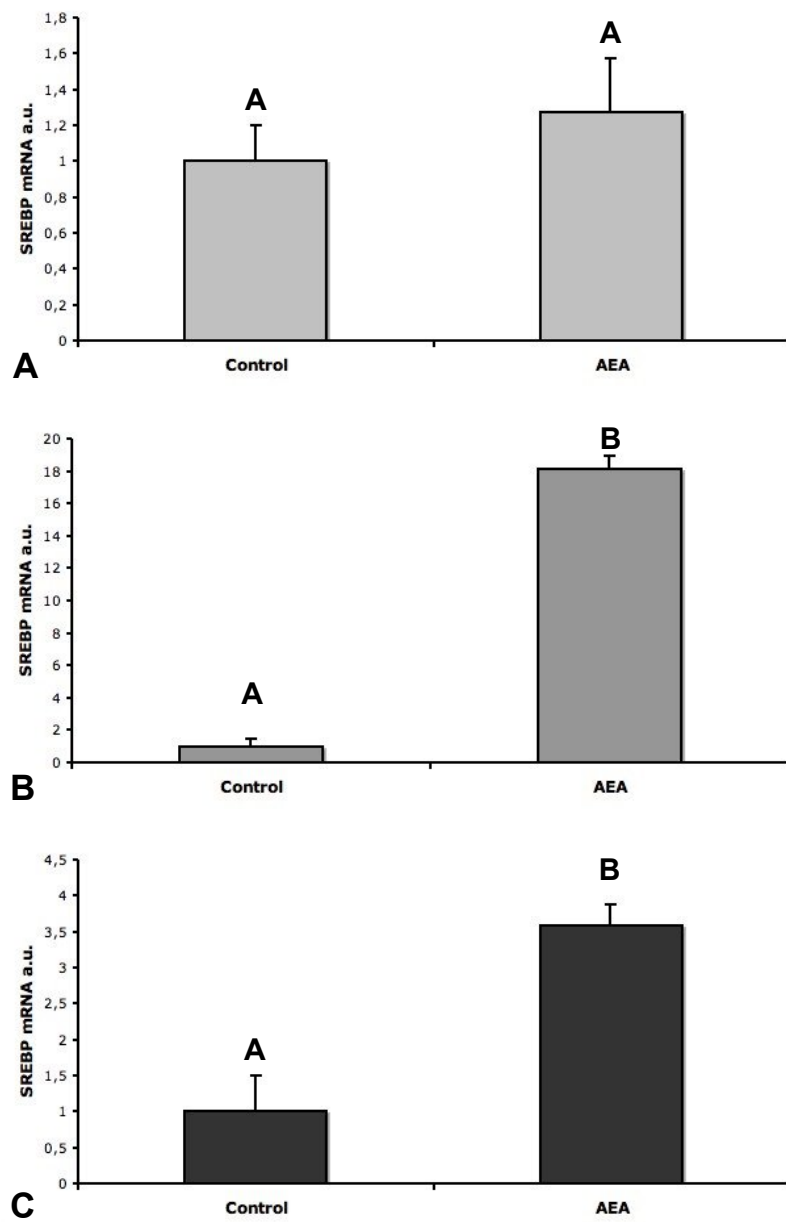


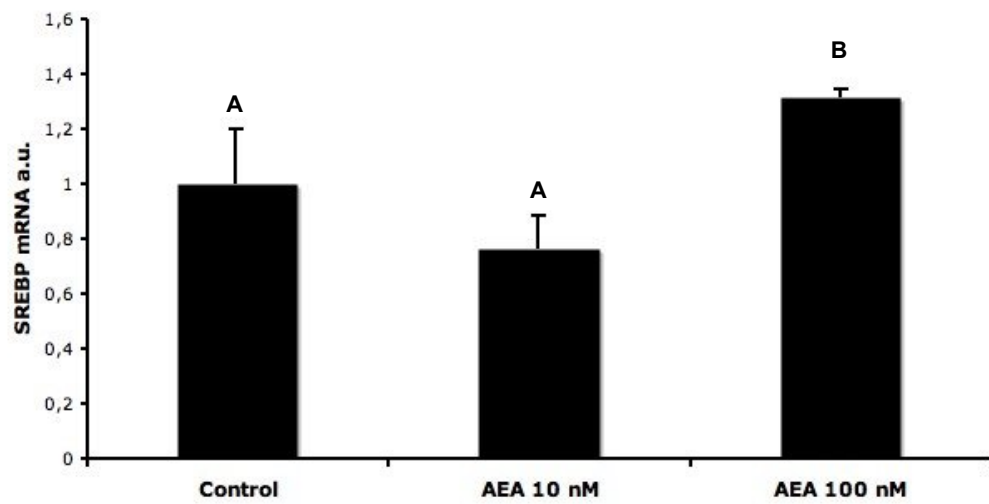
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