

ORIGINAL ARTICLE

Estrogenic induction of cannabinoid CB1 receptor in human colon cancer cell lines

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Abstract

Objective. Cannabinoids are a class of compounds that have the ability to activate two specific receptor subtypes, the cannabinoid CB1 and CB2 receptors. CB1 receptor is a G-protein-coupled receptor that is linked to the signal transduction pathways. The cumulative effects of this receptor have important implications in the control of cell survival and cell death having the potential to regulate tumor cell growth. In this connection, interest has been focused on factors such as sex steroid hormones, which regulate CB1 receptor expression. The aim of this study was to investigate the effects of 17 β -estradiol exposure on the CB1 receptor gene and its protein expression in human primary tumor colon cancer cell lines, such as DLD-1, HT-29 and one lymph node metastatic cell line, SW620. **Material and methods.** CB1 gene expression was determined using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in DLD-1, HT-29 and SW620 cells treated at different times and doses of 17 β -estradiol exposure. CB1 protein expression was detected by Western immunoblot. **Results.** 17 β -estradiol induced CB1 gene expression in all the human colon cancer cells studied. The early induction of CB1 receptor mRNA in DLD-1 and SW620 cells was mediated by the estrogen receptor because the pure estrogen antagonist, ICI 182,780, was able to counteract this effect. Estrogenic induction of the CB1 receptor was also detectable at protein level in all cell types tested. **Conclusions.** The CB1 receptor can be considered an estrogen-responsive gene in DLD-1, HT-29 and SW620 cells. Up-regulation of CB1 expression by 17 β -estradiol is a further mechanism of estrogens to control colon cancer proliferation.

Key Words: *Cannabinoid CB1 receptor, colon cancer, estradiol, human colon cell lines*

Introduction

Plant-derived, synthetic or endogenous cannabinoids are a class of compounds that have the ability to activate two specific receptors subtypes, the cannabinoid CB1 and CB2 receptors. CB1 receptors, first detected in brain, have also been detected in reproductive tissues and the gastrointestinal system [1,2], whereas CB2 receptors appear to be expressed mainly by cells of the immune system [2]. The CB1 receptor is a G-protein-coupled receptor that is linked to signal transduction pathways including inhibition of adenylate cyclase, activation of mitogen-activated protein kinase and regulation of

calcium and potassium channels [2]. The cumulative effects of this receptor have important implications in the control of cell survival and cell death having the potential to regulate tumor cell growth.

Recent studies provided good evidence to show that the major endocannabinoid, anandamide (arachidonylethanolamide), may play a role in controlling colorectal cancer cell proliferation by acting at cannabinoid receptor [3–7]. Anandamide was found to inhibit, mostly via the CB1 receptor, the proliferation of breast, prostate and colorectal cancer cells *in vitro* [7–9] and of rat v-k-ras-transformed thyroid cells by blocking the activity of p21^{ras} [5]. There is also a growing body of evidence to show

that either the selective CB1 receptor stimulation or activation of both CB1 and CB2 receptors causes inhibition of cancer growth *in vitro* [8]. These anti-proliferative effects are reversed by SR141716A, a specific antagonist for the CB1 receptor [10], which suggests a role for this receptor subtype in modulating cell proliferation.

In this connection, interest has been focused on factors regulating CB1 receptor expression. Sex steroids have been shown to regulate CB1 receptor expression in the human anterior pituitary gland [11] and it has still to be elucidated whether the same action of estrogens can occur also in the colon, on classic estrogen responsive tissue. The presence of appreciable amounts of cannabinoid receptors, as well as estrogen receptor expression, has already been established in colorectal carcinoma and normal colonic epithelium [1,2,7,12]. Moreover, clinical and experimental evidence has shown a link between sex steroid hormones and colorectal tumor showing a significant anti-proliferative action of estrogens in colon cancer [12–17]. Estradiol seems to be able to interact with molecules required for cell proliferation such as polyamine [18] and growth factors [19] in colon cancer, eliciting its effects also through the down-regulation of HMG-CoAR activity associated with the early induction of low-density lipoprotein (LDL) receptors [20].

In an effort to elucidate another aspect of estrogenic cell growth control in colon carcinoma, we investigated the effects of 17 β -estradiol exposure on CB1 receptor gene and its protein expression in human primary tumor colon cancer cell lines, such as DLD-1, HT-29 and one lymph node metastatic cell line, SW620. Changes in CB1 receptor gene expression, induced by estrogens, might be relevant owing to the considerable interest in the possible role of CB1 receptors and their endogenous ligands as cell proliferation mediators.

Material and methods

Cell culture conditions

The human colon cell lines DLD-1, HT-29 and SW620 were obtained from the ICLC (IST, Genoa, Italy). DLD-1, HT-29 and SW620 were routinely grown in Roswell Park Memorial Institute (RPMI) 1640, McCoy's 5A and Dulbecco-modified Eagle medium (D-MEM), respectively. The media, without phenol red, were supplemented with 10% fetal bovine serum (FBS), 1% non-essential aminoacids (NEAA), 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, in monolayer cultures, and incubated at 37°C in a humidified atmosphere containing 5% CO₂. At confluence, the grown cells

were harvested by means of trypsinization and serially subcultured with a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich, Milan, Italy.

17 β -estradiol treatment

In the experiments investigating the effect of 17 β -estradiol under estrogen-depleted culture conditions, cell lines were plated at a density of 2×10^5 cells/5 ml phenol red-free appropriate medium, containing 10% FBS in 60-mm tissue culture dishes (Corning Costar, Cambridge, Mass., USA). After 24 h, the medium was replaced with fresh medium containing 5% dextran-coated charcoal-stripped FBS with 17 β -estradiol, at concentrations of 0.1 nM, 1 μ M and 10 μ M dissolved in dimethyl sulfoxide (DMSO) and maintained for 1, 6 and 24 h. Charcoal-stripped FBS selectively abolished estrogens, with no variation in the sterol concentration.

Each experiment included an untreated control and a control with the same concentration of DMSO as had been used for adding estrogen. In all experiments, the solvent did not exceed a concentration of 0.3%. Quadruplicate cultures were set up for each hormone concentration and for controls, and each experiment was repeated four times in separate samples. Cell viability, determined with the trypan blue exclusion method, always exceeded 90%.

Reverse transcriptase and quantitative polymerase chain reaction (real-time PCR)

Each cell line, cultured with 0.1 nM, 1 μ M and 10 μ M of 17 β -estradiol for 1, 6 and 24 h, was washed twice in phosphate buffered saline (PBS) and then trypsinized and centrifuged at low speed. The cell pellets were resuspended in 0.3 ml pure distilled water and used for RNA extraction.

Total cell RNA was extracted using Tri-Reagent (Mol. Res. Center Inc., Cincinnati, Ohio, USA), following the manufacturer's instructions. About 2 μ g total cell RNA, extracted from both the control and treated cells, was used for cDNA synthesis. Reverse transcription (RT) was carried out in 20 μ l of the final volume at 41°C for 60 min, using 30 pmol antisense primer (Table I) for analysis of the CB1 receptor gene. The β -actin gene was utilized as an internal control and was chosen as a reference gene because it is a housekeeping gene and its expression is not regulated by estrogen [21].

Real-time PCRs were performed in 25 μ l final volume containing 2 μ l cDNA, master mix with SYBR Green (iQ SYBR Green Supermix; Bio-Rad, Milan, Italy) and sense and antisense primers for the CB1 gene and β -actin gene (Table I).

Table I. Sequences of amplification primers.

Gene		Primer
CB1 receptor	Sense	5'-GGAGAACATCCAGTGTGGGG-3'
	Antisense	5'-CATTGGGGCTGTCTTTACGG-3'
β -actin	Sense	5'-AAAGACCTGTACGCCAACACAGTGCTGTCTGG-3'
	Antisense	5'-CGTCATACTCCTGCTTGCT GATCCACATCTGC-3'

Real-time PCR was carried out with iCycler Thermal Cycler System apparatus (Bio-Rad) using the following parameters: one cycle of 95°C for 1 min and 30 s, followed by 45 cycles at 94°C for 10 s, 55°C for 10 s and 72°C for 30 s and a further melting curve step at 55–95°C with a heating rate of 0.5°C per cycle for 80 cycles. The PCR products were quantified by external calibration curves, obtained with serial dilutions of a known copy number of molecules (10^2 – 10^7 molecules). All expression data were normalized with expression of the house-keeping gene β -actin used as the internal control. The specificity of the PCR product of each tested gene was confirmed by gel electrophoresis.

Western blot analysis

For CB1 receptor protein evaluation, immunoblot analysis was carried out in DLD-1, HT-29 and SW620 cells treated with 0.1 nM, 1 μ M and 10 μ M of 17 β -estradiol for 1, 6 and 24 h. Parallel experiments were conducted in untreated control cells. After estrogen treatment, the cells were washed twice in PBS and pelleted by centrifugation. Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.2; 1 mM phenylmethylsulfonylfluoride; 2 μ M aprotinin; and 10 μ M leupeptin), disrupted by sonication and centrifuged at 3000 rpm for 10 min at 4°C. Protein (50 μ g) cell supernatants were electrophoresed on 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and blocked with 7.5% milk in Tris-buffered saline-Tween 20 for 1 h at room temperature. The filters were then probed with goat polyclonal IgG anti-human CB1 receptor (Santa Cruz Laboratories) at a 1:1000 dilution. Immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated donkey anti-goat IgG (BioRad, Hercules, Calif., USA), using the enhanced chemiluminescence

system (ECL, Amersham, Buckinghamshire, UK). CB1 receptor protein was expressed as the value of optical density (OD).

Statistical analysis

Data were statistically evaluated by ANOVA followed by the Dunnett Multiple Comparison Test. Differences were considered significant at a 5% probability level.

Results

The cell line origins and CB1- or estrogen receptor basal expressions of DLD-1, HT-29 and SW-620 cell lines are presented in Table II.

In the metastatic colon cancer cell line SW620 the CB1 receptor mRNA basal levels were higher than those in the other two cell lines (Figure 1). In these human colon cancer cells the CB1 receptor is subjected to a positive influence exerted by treatment with 17 β -estradiol (Figure 2). Compared with control cells, there was a significant induction of CB1 receptor mRNA after 1 h of hormone administration, at 0.1 nM ($p < 0.05$, Dunnett's post-test) in SW620 cells (Figure 2C). A higher concentration (10 μ M) of 17 β -estradiol was necessary to induce CB1 receptor mRNA in DLD-1 and HT-29 after 1 h (Figure 2A, B). After 6 h of treatment, the significant induction was detectable at 0.1 nM, 1 μ M and 10 μ M ($p < 0.05$, Dunnett's post-test) in DLD-1, HT-29 and SW620. After 24 h, the induction of CB1 receptor mRNA reached statistical significance only in HT-29 cells.

To evaluate whether the effect of 17 β -estradiol on CB1 mRNA expression is mediated through the estrogen receptor, cells were also exposed to 0.1 nM 17 β -estradiol for 6 h with or without 10 nM ICI 182,780 (a pure estrogen receptor antagonist). As shown in the Figure 2A and 2C, ICI 182,780

Table II. Cell line origins and CB1-or estrogen receptor expression profiling.

Cell type	Origin	CB1 receptor	Estrogen receptor α	Estrogen receptor β
DLD-1	Colorectal adenocarcinoma	Positive	Negative	Positive
HT-29	Colorectal adenocarcinoma grade II	Positive	Negative	Negative
SW620	Lymph node metastasis	Positive	Positive	Positive

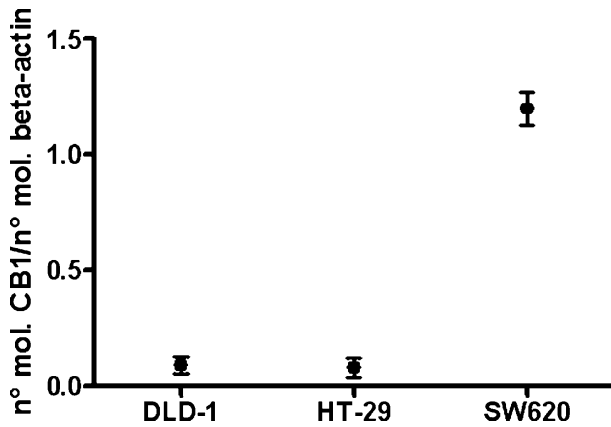


Figure 1. CB1 receptor mRNA basal levels in DLD-1, HT-29 and SW620 cell lines.

completely abolished the stimulation of CB1 receptor gene in DLD-1 and SW620. No effect was exerted by ICI 182,780 in HT-29 cells (Figure 2B).

Figure 3 delineates the densitometric analysis of the CB1 receptor protein expression in DLD-1, HT-29 and SW620 cells after exposure at 0.1 nM, 1 μ M and 10 μ M of 17 β -estradiol for 1, 6 and 24 h. A significant protein induction exerted by 1 h of estrogenic treatment was detectable at all concentrations of hormone (0.1 nM and 1 μ M and 10 μ M) in SW620 and was kept until 6 h and 24 h (Figure 3C). An increase in CB1 receptor protein levels starting from 10 μ M after 1 h of 17 β -estradiol was evident in DLD-1 and HT-29, and at concentrations of 0.1 nM and 1 μ M the induction was present after 6 h of treatment (Figure 3A, B).

Discussion

The present study provides additional information on the mechanisms by which estrogens can modulate colon cancer proliferation. We have previously demonstrated the inhibitory effect of estrogens on gastric cell lines [18,19]. The exposure of a human estrogen receptor-positive gastric cancer cell line to progressive increasing 17 β -estradiol concentrations caused a significant anti-proliferative action and an enhanced apoptotic rate, in a dose-dependent manner. We have also shown that the estradiol is able to interact with molecules like polyamine [18] and growth factors [19] required for cell proliferation. Recently, we have demonstrated an estrogenic regulation of cholesterol biosynthesis and cell growth in DLD-1 human colon cancer cells, supporting the idea that the cholesterol metabolism could be considered another target for the estrogenic anti-proliferative properties [20].

In the present study we found that 17 β -estradiol induces CB1 gene expression in human colon cancer

cells, showing over-expression of the CB1 receptor after short exposure to estrogens.

Our results indicate that transcriptional activation of CB1 after estradiol treatment could be a direct estrogen receptor-mediated effect, at least in DLD-1 and SW620 cell lines. To date, we have no knowledge of any estrogen response elements (ERE) in the CB1 promoter, and the putative regulative region of this gene is not well known.

Moreover, based on the presented evidence, the CB1 receptor can be considered an estrogen-responsive gene in DLD-1, HT-29 and SW620 cells. The early induction of CB1 receptor mRNA in DLD-1 and SW620 cells is mediated by the estrogen receptor because the pure estrogen antagonist ICI 182,780 was able to counteract this effect. No effect of ICI 182,780 was detectable in HT-29 cells, known to be estrogen receptor negative cells. We suggest that in these cells the hormone could exert its effects by different mechanisms, potentially acting through a direct interaction with estrogen-responsive genes.

Estrogenic induction of the CB1 receptor was also detectable at protein level in all cell types tested. After 24 h of hormone treatment, a statistically significant induction of protein levels was present only in SW620 cells. In all probability, this effect was due to the presence of both estrogen receptors α and β that keep the protein induction at high levels in this cell line.

The present findings suggest that the anti-proliferative action of 17 β -estradiol on human colon cancer cells may also be related to its ability to induce CB1 receptor expression. Estrogenic regulation of the CB1 receptor gene in colon cancer cell lines, detected in this study, concurs with the results of recent studies showing that both CB1 receptor gene expression and the FAAH enzyme are influenced by estrogen in the human anterior pituitary gland and in mouse endometrial epithelium, respectively [11,22].

Up-regulation of the endocannabinoid system by estrogens is a further mechanism of growth control in colon cancer; therefore, the use of estrogens in an anticancer therapeutic strategy has to be encouraged. Finally, the activation of synthesis of endocannabinoids and their receptors, as well as the inactivation of their enzymatic hydrolysis might be important in a strategy for developing new anticancer drugs.

Acknowledgements

We thank Benedetta D'Attoma and Valeria Tutino for excellent technical assistance. This work was supported by a grant from the Ministero della Salute no. ICS-160.2/RF99.65 and by the Associazione Educazione e Ricerca Medica Salernitana (ERMES).

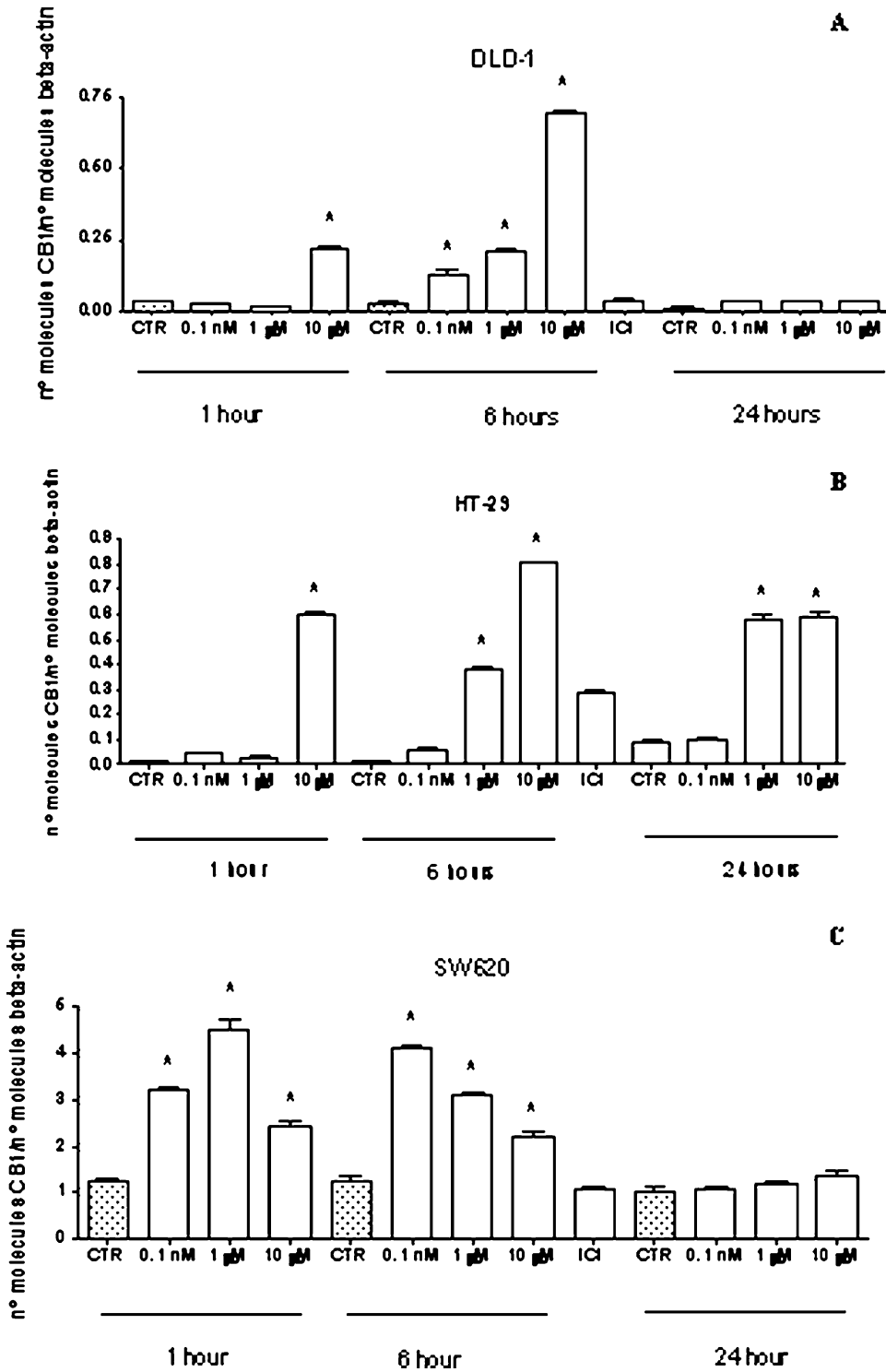


Figure 2. Effects of 0.1 nM, 1 μM and 10 μM of 17β-estradiol on CB1 receptor mRNA levels of DLD-1 (A), HT-29 (B) and SW620 (C) cells cultured for 1 h, 6 h and 24 h. The effect of 0.1 nM 17β-estradiol in the presence of 10 nM ICI 182,780 after 6 h of treatment is also shown. Data displayed as mean values ±SE. The *p*-value was determined by one-way analysis with Dunnett's post-test; **p* < 0.05 versus control.

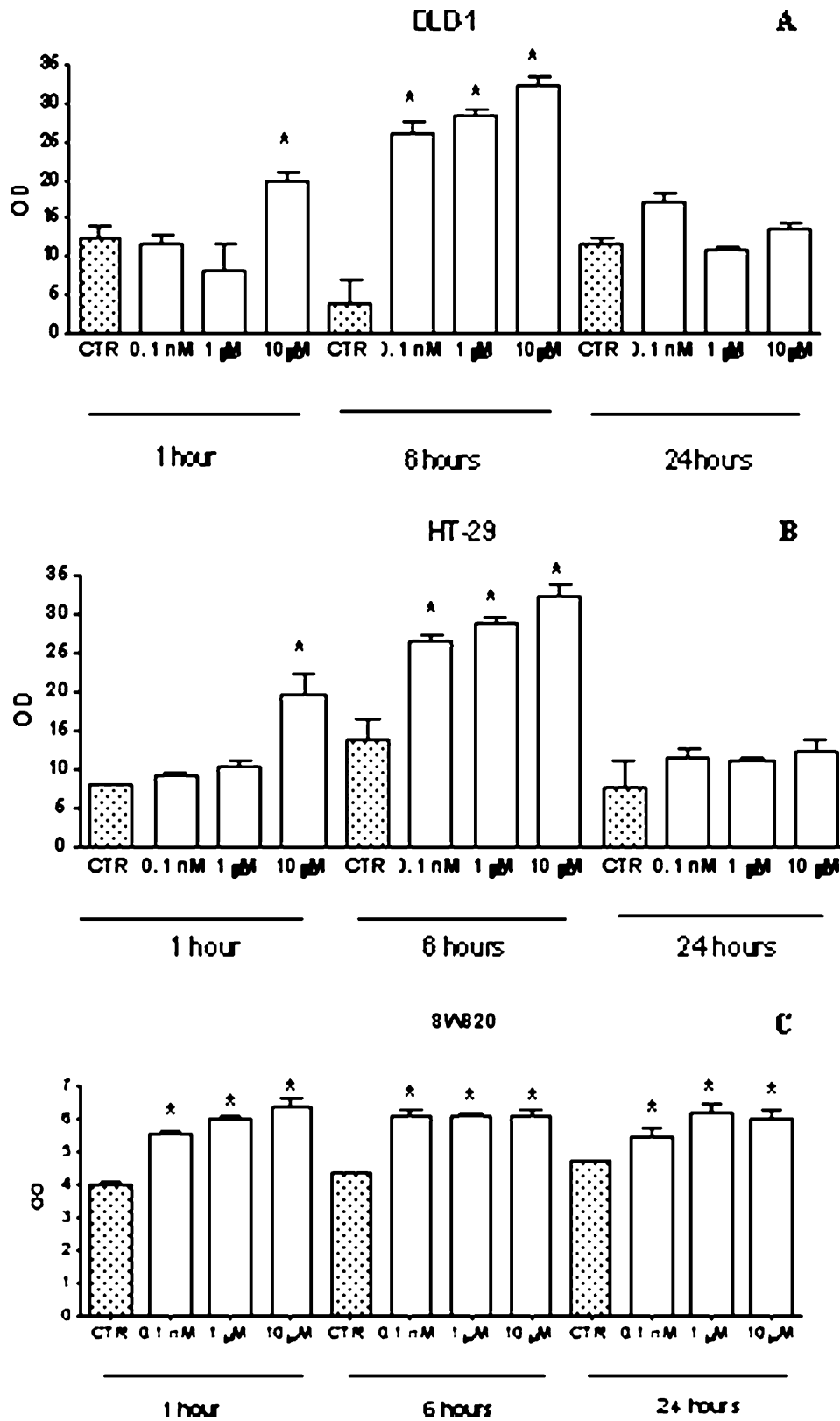


Figure 3. Densitometric analysis of CB1 protein levels (OD) after 17β-estradiol treatment (0.1 nM, 1 μM and 10 μM) for 1 h, 6 h and 24 h in DLD-1 cells (A), HT-29 (B) and SW-620 (C). Results are expressed as mean values ±SE. The *p*-value was determined by one-way analysis with Dunnett's post-test; **p* < 0.05 versus control.

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