Antiproliferative effects of β-blockers on human colorectal cancer cells

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Abstract. Colon cancer is the fourth and third most common cancer, respectively in men and women worldwide and its incidence is on the increase. Stress response has been associated with the incidence and development of cancer. The catecholamines (CA), adrenaline (AD) and noradrenaline (NA), are crucial mediators of stress response, exerting their effects through interaction with α- and β-adrenergic receptors (AR). Colon cancer cells express β-AR, and their activation has been implicated in carcinogenesis and tumor progression. Interest concerning the efficacy of β-AR blockers as possible additions to cancer treatment has increased. The aim of this study was to investigate the effect of several AR agonists and β-blockers following cell proliferation of HT-29 cells, a human colon adenocarcinoma cell line. For this purpose, HT-29 cells were incubated in the absence (control) or in the presence of the AR-agonists, AD, NA and isoprenaline (ISO) (0.1-100 μM) for 12 or 24 h. The tested AR agonists revealed proliferative effects on HT-29 cells. In order to study the effect of several β-blockers following proliferation induced by AR activation, the cells were treated with propranolol (PRO; 50 μM), carvedilol (CAR; 5 μM), atenolol (ATE; 50 μM), or ICI 118,551 (ICI; 5 μM) for 45 min prior, and simultaneously, to incubation with each of the AR agonists, AD and ISO, both at 1 and 10 μM. The results suggested that adrenergic activation plays an important role in colon cancer cell proliferation, most probably through β-AR. The β-blockers under study were able to reverse the proliferation induced by AD and ISO, and some of these blockers significantly decreased the proliferation of HT-29 cells. The elucidation of the intracellular pathways involved in CA-induced proliferation of colon cancer cells, and in the reversion of this effect by β-blockers, may contribute to identifying promising strategies in cancer treatment.

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Introduction

Colon cancer is a leading cause of cancer and cancer-related mortality in the Western world, and its incidence is on the increase (1). Stress has been associated with the increased incidence and development of cancer (2). Adrenaline (AD) and noradrenaline (NA), two of the most important mediators released in response to stress, exert their effects through interaction with α- and β-adrenergic receptors (ARs). ARs are targets for many therapeutically important drugs, such as the ones used for cardiovascular diseases, asthma, prostatic hypertrophy, nasal congestion obesity and pain (3). Expression of β-AR has been identified in colon cancer cells and previous studies have shown that their activation has been implicated in carcinogenesis and tumor progression (4-6). Interest regarding the efficacy of β-AR blockers as possible additions to cancer-treatment paradigms has increased. However, which of these drugs are useful remains to be determined.

Previous findings suggest that drug efficacy may be influenced by the signaling effectors engaged by a unique receptor (7). For instance, some β-AR blockers that are inverse agonists for G-protein-mediated functions were found to be agonists, neutral blockers, or inverse agonists for β-arrestin-mediated signaling, resulting in markedly different effects *in vivo* (7). A better description of the efficacy profiles for β-AR blockers may be useful to explain the reason for individual members of a drug class having different therapeutic indications.

Over the last years, the biological effects of stress pathways on cancer progression have been focused on the effects of stress hormones on tumor cell proliferation, apoptosis, invasion, metastasis, angiogenesis, stroma-cell microenvironment and cellular immune responses (8). Several *in vitro* and *in vivo* studies have shown that AD and NA can induce cell proliferation in different types of cancer such as non-small cell lung carcinoma (9,10), colon cancer (5), oral squamous carcinoma (11), breast cancer (12) and prostate cancer (13).

In the present study, we aimed to clarify the role of AD, NA and ISO, and several β-blockers on colon-cancer cell proliferation using a human colon adenocarcinoma cell line.

Materials and methods

Reagents. RPMI-1640 medium was purchased from Invitrogen (Invitrogen Life Technologies, Paisley, UK). The following reagents were purchased from Sigma (St. Louis, MO, USA): AD (Adrenaline-L-adrenaline(+)-bitartrate salt), NA (Noradrenaline-L-(-)-noradrenaline(+)-bitartrate salt monohydrate), ISO (Isoprenaline-(-)-isoprenaline(+)-bitartrate salt), PRO (Propranolol-DL-propranolol hydrochloride), ICI-(±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanolhydrochloride) (ICI 118,551), ATE (Atenolol-(±)-4-[2-hydroxy-3[(1methylethyl)amino]propoxy]benzeneace tamide), penicillin, streptomycin, (FBS) fetal bovine serum, trypsin-EDTA solution and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. CAR {Carvedilol-1-(9H-carbazol-4-yloxy)-3-[(2-(2-methoxyphenoxy)ethyl) amino]-2-propanol} was purchased from Enzo Life Sciences, Inc., (Farmingdale, NY, USA). The cell proliferation ELISA BrdU kit (colorimetric) was purchased from Roche Diagnosis GmbH (Mannheim, Germany) and Cell Titer 96® Aqueous ONE Solution Reagent cell proliferation assay (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] were purchased from Promega (Madison, WI, USA).

Cell culture. HT-29 human colon adenocarcinoma cells were kindly provided by Professor Bruno Sarmento (Institute of Biomedical Engineering-INEB) and Professor Fernando Magro (Faculty of Medicine of the University of Porto).

HT-29 cells were cultured in RPMI-1640 medium supplemented with 10% of FBS, 100 U/ml penicillin and 100 μg streptomycin. The cells were grown at 37˚C in a humidified 5% CO_2 atmosphere. Culture medium was changed every 2-3 days. When the cells reached 90-100% confluency, the medium was removed, and the cell monolayer was washed once with PBS. The cell monolayer was treated with 1 ml of 0.25% (w/v) trypsin-EDTA and incubated for 2 min to ensure complete cell detachment. For sub-culturing, the cells were sub-cultured in plastic culture dishes (21 cm^2, 60-mm diameter, Corning Costar, Corning, NY, USA). For the experiments, HT-29 cells were seeded in 96-well (0.37 cm^2, 6.9 mm diameter, TPP) or 24-well plastic cell culture clusters (2 cm^2, 16-mm diameter, TPP) depending on experimental conditions. Experiments were performed 4-5 days after the initial seeding (90-100% confluency).

Viability experimental studies

Trypan blue exclusion assay. Prior to any experiment, cell viability was determined by Trypan Blue exclusion assay. The experiments were only performed, when viability was >90%. In brief, cells were trypsinized and stained with 0.4% trypan blue and viable cells were counted with a hemocytometer.

MTS assay. HT-29 cells were seeded at $1x10^5$ cells/ml in 96-well plates for 24 h and incubated with each treatment for 12 or 24 h, depending on experimental conditions. Cell viability assay was assessed using Cell Titer 96 Aqueous ONE Solution Reagent cell proliferation assay (MTS) according to the manufacturer's instructions. Briefly, the culture medium was removed and the cells were pre-incubated with the compounds under study in culture medium at 37˚C for 12 or 24 h. This medium was removed and the cells were incubated for 3 h with 100 μl of FBS-free culture medium and 20 μl of MTS. Optical density was measured at 492 nm. Results were expressed as percentage of the control.

MTT assay. In order to determine the half maximal inhibitory concentration (IC_{50}), the concentration that reduces the effect by 50%, and the half maximal effective concentration (EC_{50}) values, the concentration that yields half maximal response, an MTT assay was performed. This method is based on mitochondrial dehydrogenase activity. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals insoluble compounds in aqueous solutions. The amount of these compounds was determined spectrophotometrically. The cells were seeded in 96-well plates at a density of $1x10^4$ cells/well for 24 h, and then incubated for 24 h with increasing concentrations of the various compounds under study (0.1, 1, 5, 10, 20, 50 and 100 μM). The cells were then washed twice with PBS and incubated with MTT (5 mg/ml) for 2 h at 37˚C. Blue formazan crystals were solubilized with DMSO and the colored solution was subsequently read at 550 nm. The samples were assayed in triplicate and at least in three independent experiments, and the mean value for each experiment was calculated. Results were presented as mean (± SEM) and are expressed as percentage of the control [adapted from Stanojcovic *et al* (14)].

Proliferation experimental studies. Cell proliferation was assessed as DNA synthesis. To evaluate DNA synthesis, the incorporation of [^{3}H]-thymidine or 5'-bromodeoxyuridine (BrdU) into DNA was determined, as detailed in the subsequent sections.

Incorporation of [^{3}H]-thymidine. HT-29 cells were seeded for attachment at $5x10^4$ cells/well in 24-well (1.65 cm^2, 14.5 mm diameter; Orange Scientific, Belgium) plastic cell-culture clusters in a final volume of 0.5 ml culture medium containing 10% FBS. After 24 h in culture, the cells were treated with several concentrations of the adrenergic agonists dissolved in culture medium (controls were produced in the presence of culture media). After 24 h, the cells were incubated with 0.2 ml of methyl-[^{3}H]-thymidine (0.5 μCi/well) for 4 h. The medium was removed and the cells were fixed by incubation in 0.3 ml of 10% TCA for 1 h at 4˚C. The cells were then washed twice with 0.3 ml of 10% TCA to remove unbound radioactivity. The plates were air-dried and the cells lysed with 0.28 ml/well of 1 M NaOH. A 0.25-ml aliquot of the lysate was neutralized with 0.050 ml of HCl prior to the addition of scintillation fluid. Radioactivity of the samples was quantified by a liquid scintillation counter. The counts (disintegrations/min) of each treatment were averaged and expressed as percentage of the controls [adapted from Miranda *et al* (15)].

Incorporation of BrdU. The incorporation of the BrdU assay is a method based on the incorporation of BrdU, a thymidine analogue, instead of thymidine into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by immunoassay.

HT-29 cells were grown at $1x10^5$ cells/ml in 96-well plates for 24 h, and proliferation was measured using the Cell Proliferation ELISA BrdU kit (Roche Diagnostics GmbH), according to the manufacturer's instructions. Briefly, the cells were labeled with BrdU at a final concentration of 10 μM/well),

Table I. EC_{50} values for adrenaline and isoprenaline on HT-29 proliferation.

Cell type	Agonist	$EC_{50}/\mu M$	95% CI	n
HT-29	Adrenaline	9.98	0.51-197.2	10-11
	Isoprenaline	29.27	0.72-1,194.0	8-12

EC_{50}, half maximal effective concentration; CI, confidence interval.

Table II. IC_{50} of β-blockers on HT-29 proliferation.

Cell type	β-blockers	$IC_{50}/\mu M$	95% CI	n
HT-29	Propranolol	65.4	33.7-126.9	10-12
	Carvedilol	8.0	6.0-10.6	12
	Atenolol	52.9	21.7-128.7	6-12
	ICI 118,551	8.9	6.5-12.0	12

IC_{50}, half maximal inhibitory concentration; CI, confidence interval.

for 12 h at 37˚C. The cells were then denatured with FixDenat solution, and incubated for 120 min with 1:100 diluted mouse anti-BrdU conjugated to peroxidase. Following removal the antibody conjugate and washing twice with washing solution (PBS 1X), the substrate solution was added for 25 min and, after this period, the reaction was stopped with 1 M H_2SO_4 solution. Absorbance was measured within 5 min at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader. The blank corresponded to 100 μl of culture medium without BrdU, and the control was produced in the presence of culture media without any treatment. Results were presented as mean (± SEM) and are expressed as percentage of the control.

Another protocol for the BrdU assay was tested for treatments at 24 h. Briefly, $5x10^3$ cells/well were seeded in 96-well plates. The medium was supplemented with antibiotics plus 1% FBS for cell attachment. The cells were subsequetly starved in serum-free medium for another 12 h to synchronize the cell cycle. HT-29 cells were incubated with AD, ISO or NA (0, 1 and 10 μM) for 24 h to study the growth-promoting effect of these adrenergic agonists. To examine the effects of various β-blockers, the cells were pretreated with or without, PRO (50 μM), CAR (5 μM), ATE (50 μM) and ICI (5 μM) for 45 min prior to, and also simultaneously with, AD or ISO treatment. Cell proliferation was indicated by the amount of DNA synthesis measured with the BrdU incorporation assay kit, according to the manufacturer's instructions. Briefly, the cells were labeled with 10 μl/well BrdU and incubated at 37˚C for 4 h. Following removal of the labeling medium, the cells were fixed and probed with the anti-BrdU monoclonal antibody at 25˚C for 2 h and its substrate tetramethyl-benzidine (TMB) at 25˚C for 30 min. After removal of the unconjugated antibody, the cells were rinsed three times with the washing solution and treated with 200 μl/well substrate solution. After color development, 1 M H_2SO_4 was added (25 μl/well) to stop the substrate reaction, and the absorbance of each sample was measured in an enzyme-linked immunosorbent assay (ELISA) microplate reader at 450 nm (with a reference wavelength at 690 nm for blank to disccount the non-specific binding to the anti-BrdU antibody). The value from the non-specific binding was subtracted from all the other values. The results were presented as mean (± SEM) and expressed as percentage of the control.

Statistical analysis. The results were presented as arithmetic mean ± SEM. Differences in cell proliferation, viability or cell growth between treated and corresponding untreated cells (controls) were tested using Student's t-tests. For the calculation of EC_{50} and IC_{50} values, the parameters of the Hill equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method (15), with 'n' representing the number of replicates of at least three different experiments. Comparisons between ≥3 groups were performed with one-way analysis of variance (ANOVA) followed by the Tamhane or Bonferroni test. Differences were considered statistically significant when $P<0.05$.

Given the variability of the results on different days, each experimental finding was adjusted to the respective control.

Results

General. The initial aim of this study was to assess the viability of HT-29 cells following chronic exposure to several AR agonists/antagonists under evaluation. For this purpose, the MTS (for treatment at 12 and 24 h with the AR agonists) and MTT (for the determination of IC_{50} and EC_{50} values) assays were used. After these initial experiments, we studied the effects of the same drugs mentioned above on cellular proliferation measured by DNA synthesis.

Effect of chronic treatment with the adrenergic agonists on cell viability. The MTS assays showed that none of the tested adrenergic agonists used, at 12 or 24 h, affected the viability of HT-29 cells (Fig. 1). By contrast, for the two treatments carried out, we observed that for the majority of the concentrations the agonists enhanced cell viability.

Determination of EC_{50} values for the adrenergic agonists in HT-29 cells. Cell exposure to AD and ISO generated concentration-response curves for the two agonists (Fig. 2). Thus, the respective EC_{50} values at 9.98 (0.51-197.2) and 29.27 (0.72-1194.0) μM for AD and ISO, respectively, were calculated (Table I).

Determination of IC_{50} values for the β-blockers in HT-29 cells. PRO potently inhibited the viability of HT-29 cells at concentrations >50 μM, with 65.4 μM being the IC_{50} for this drug (Fig. 3). HT-29 viability was inhibited in a concentration-dependent manner by CAR following exposure for 24 h. Among the β-blockers tested, CAR was identified as the most potent blocker for this effect, with an IC_{50} of 8.0 μM. ATE, when used at the highest concentration (100 μM), significantly decreased HT-29 viability, with 52.9 μM being its IC_{50} value. CAR and ICI showed similar IC_{50} values, 8.0 and 8.9 μM, respectively (Table II).

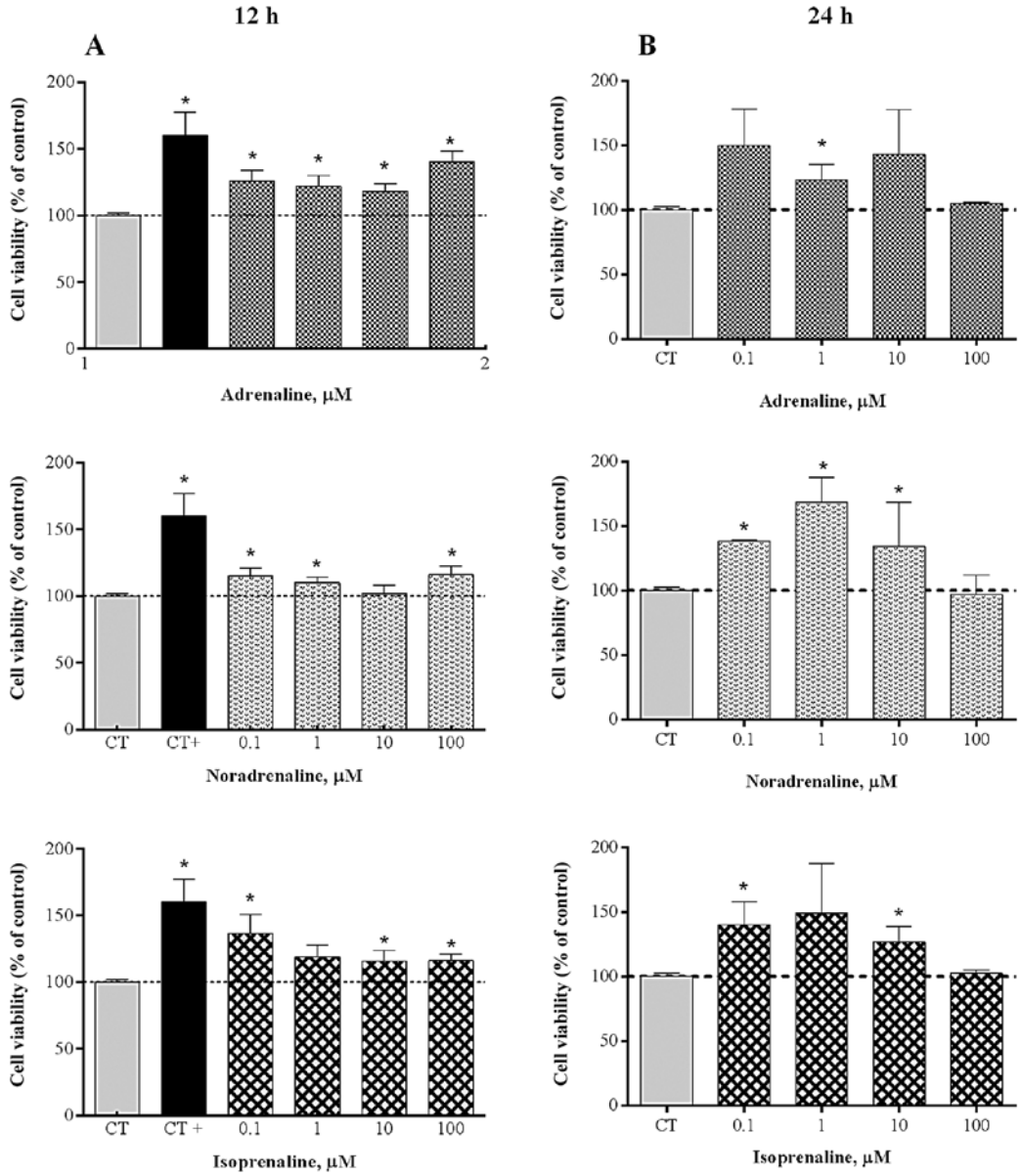

Figure 1. Effect of adrenaline (AD), noradrenaline (NA) and isoprenaline (ISO), in HT-29 human colon adenocarcinoma cells at 0-100 μM on viability assessed by MTS assay as described in the Materials and methods section, after incubation for (A) 12 h (n=11-15) and (B) 24 h (n=3-14). Results are expressed as mean ± SEM; *P<0.05 compared to the control.

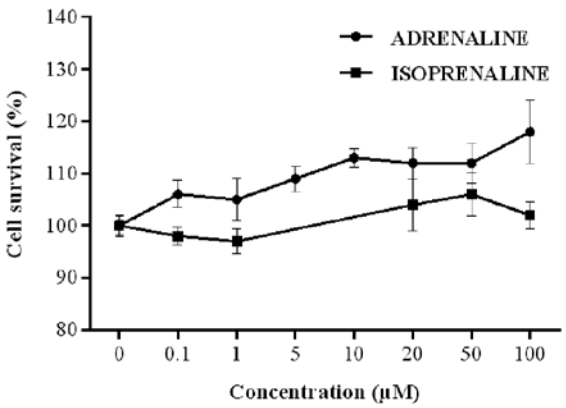

Figure 2. Effect of adrenaline and isoprenaline on the growth of HT-29 cells assessed by MTT assay after 24 h of treatment. Results are presented as mean ± SEM and are normalized to 100% of the control groups (groups without drugs).

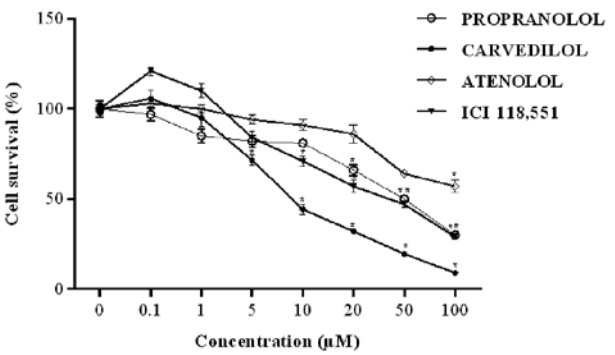

Figure 3. Concentration-response curves for HT-29 cell survival. The cells were treated with increasing concentrations (0, 0.1, 1, 5, 10, 20, 50 and 100 μM) of each β-blocker, propranolol (PRO), carvedilol (CAR), atenolol (ATE) and ICI 118,551 (ICI) for 24 h and viability was assessed by MTT assay. Results are presented as mean ± SEM and normalized to 100% of the control groups (without drugs). *P<0.05 compared to the control.

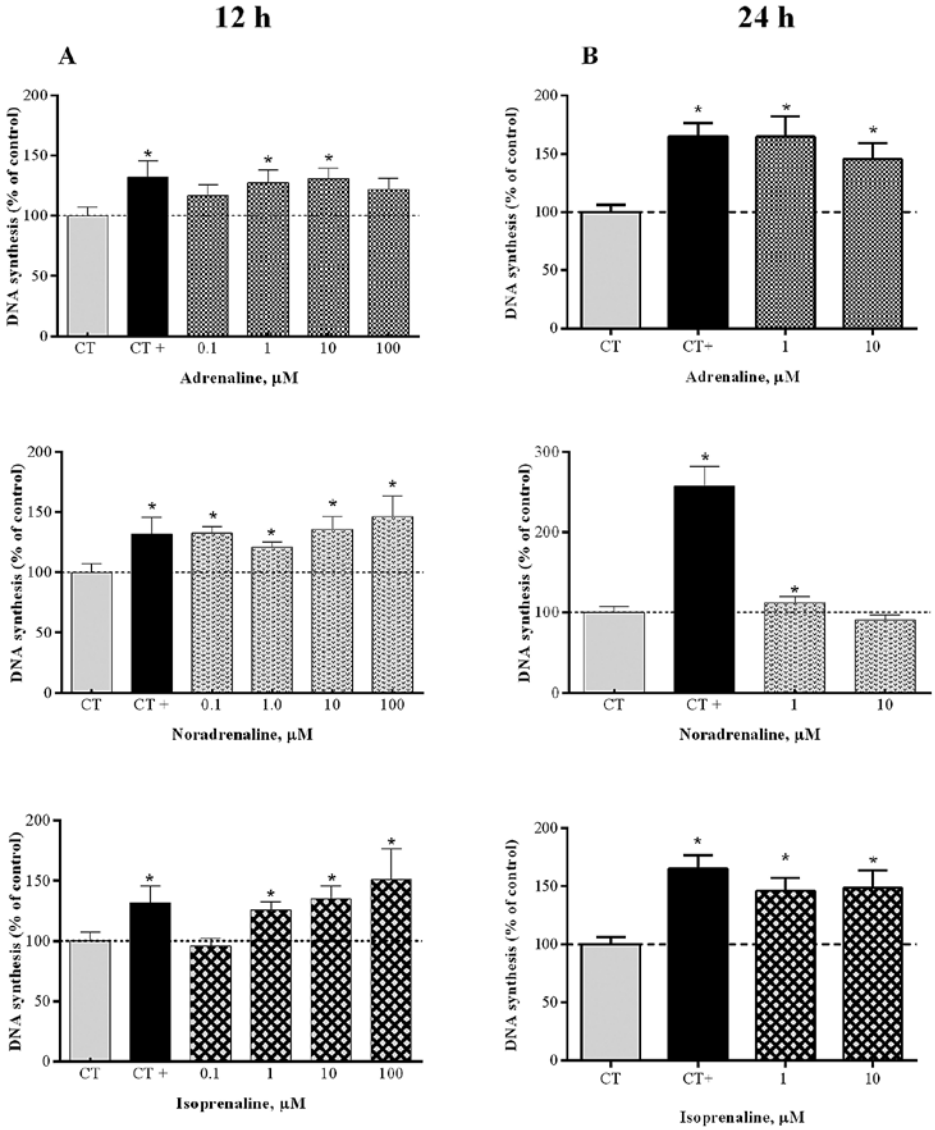

Figure 4. Effect of adrenaline (AD), noradrenaline (NA) and isoprenaline (ISO) at 0-100 μM, in HT-29 human colon adenocarcinoma cells on proliferation assessed by BrdU incorporation assay as described in Materials and methods, after incubation for (A) 12 h (n=6-12) and (B) 24 h (n=6-18). Results are presented as mean ± SEM and normalized to 100% of the control groups (without drugs). *P<0.05 compared to the control.

Effect of chronic treatment with the adrenergic agonists on HT-29 cell proliferation. The exposure of HT-29 cells to the adrenergic agonists AD, NA and ISO (at 0.1-100 μM) for 12 h markedly increased proliferation of these cells (Fig. 4A). After this period, AD had its maximum effect on proliferation at 10 μM (131.0±8.7%, n=10), NA at 100 μM (146.3±17.1%, n=8) and ISO at 100 μM (150.9±25.5%, n=7), relative to the controls. By contrast, chronic treatment for 24 h with AD led to a significant increase of cell proliferation by 164.7% (n=18) and 145.5% (n=18), when used at 1 and 10 μM, respectively (Fig. 4B), whereas ISO enhanced HT-29 cell proliferation by 146.1% (n=10) and 148.6% (n=15), respectively, at 1 and 10 μM, when compared to the controls.

Effect of β-blockers on HT-29 cell proliferation. To elucidate the role of β-AR following cell proliferation induced by AR activation, the agonists AD and ISO (a non-selective vs. a β-selective agonist) were employed following and simultaneously with the β-blockers PRO (50 μM), CAR (5 μM), ATE (50 μM) or ICI (5 μM) for 24 h. AD-induced cell proliferation was markedly reduced by PRO to 11.8±3.4% (n=6) and 32.0±9.7% (n=5), when AD was used at 1 and 10 μM, respectively. PRO markedly decreased cell proliferation stimulated by ISO to 20.1±2.1% (n=5) and 23.3±5.2% (n=5), when the agonist was used at 1 and 10 μM, respectively (Fig. 5). Furthermore, PRO induced a significant proliferation decrease to 44.2±9.6% (n=6), when compared with the control group (Fig. 5). The response profile of CAR, a potent non-selective β- and α_1-AR antagonist, in reversing the proliferative effects of AD and ISO, was similar to the results obtained with PRO. CAR was able to markedly inhibit the proliferative effect induced by the two agonists (Fig. 6). CAR decreased the proliferation induced by AD to 28% (n=6) and 56% (n=6), when AD was applied at 1 and 10 μM, respectively, and to 27% (n=6) and 36% (n=6) when ISO was used at 1 and 10 μM, respectively. In contrast to PRO, CAR did not significantly affect the proliferation of HT-29 cells. To elucidate the role of β_1-AR in HT-29 proliferation, we used ATE, a β_1- selective antagonist. Fig. 7

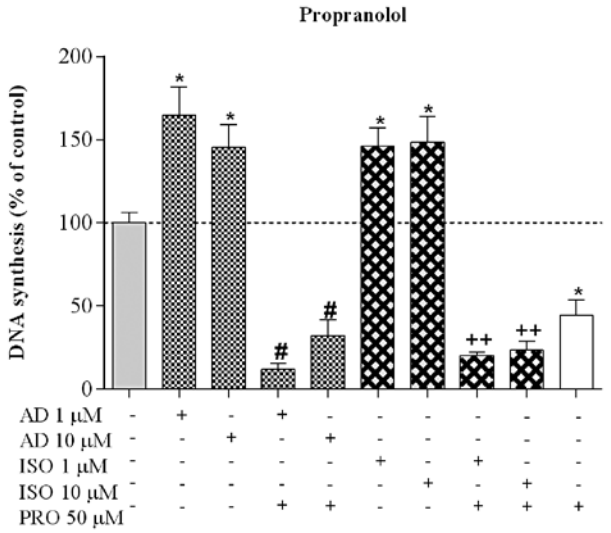

Figure 5. Effect of the non-selective β-adrenoceptor antagonist propranolol (PRO) on HT-29 cell proliferation induced by adrenaline (AD) and isoprenaline (ISO). Cells were pretreated with PRO for 45 min prior to incubation, and simultaneously with AD and ISO for 24 h. Cell proliferation was measured by BrdU incorporation assay, as described in Materials and methods. *P<0.05, significantly different from the untreated control group. #P<0.01, significantly different from the respective concentration AD-treated group and ++P<0.01 significantly different from the respective concentration ISO-treated group.

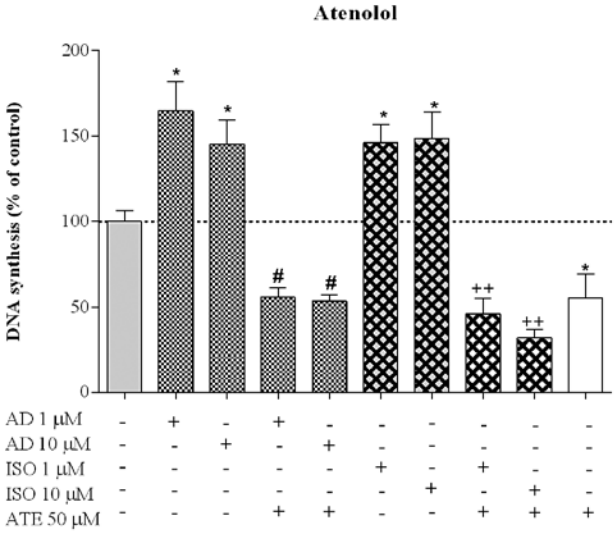

Figure 7. Effect of the selective β_1-adrenoceptor atenolol (ATE) on HT-29 cell proliferation induced by adrenaline (AD) and isoprenaline (ISO). Cells were pretreated with ATE for 45 min prior to incubation, and simultaneously with AD and ISO for 24 h. Cell proliferation was measured by a BrdU incorporation assay, as described in Materials and Methods. *P<0.05, significantly different from the untreated control group. #P<0.01, significantly different from the respective concentration AD-treated group and ++P<0.01 significantly different from the respective concentration ISO-treated group.

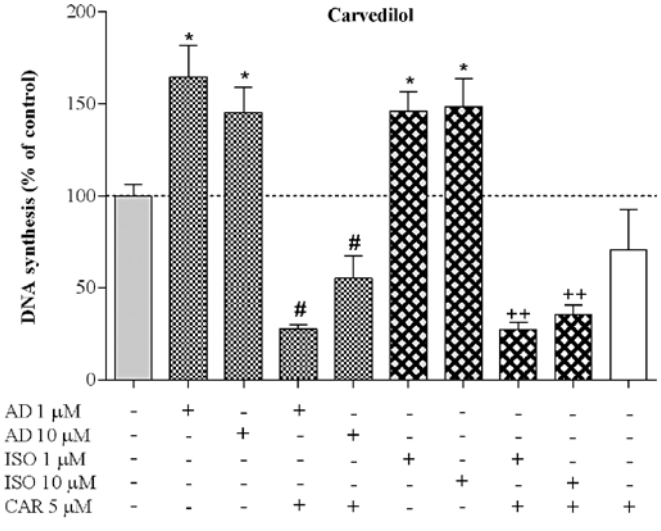

Figure 6. Effect of the non-selective β- and α_1-adrenoceptor antagonist carvedilol (CAR) on HT-29 cell proliferation induced by adrenaline (AD) and isoprenaline (ISO). Cells were pretreated with CAR for 45 min prior to incubation, and simultaneously with AD and ISO for 24 h. Cell proliferation was measured by BrdU incorporation assay, as described in Materials and Methods. *P<0.05, significantly different from the untreated control group. #P<0.01, significantly different from the respective concentration AD-treated group and ++P<0.01 significantly different from the respective concentration ISO-treated group.

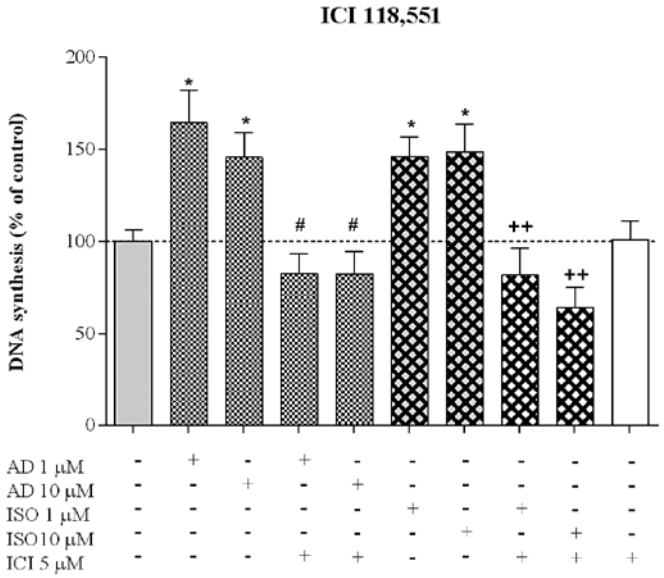

Figure 8. Effect of the selective β_2-adrenoceptor ICI-118,551 (ICI) on HT-29 cell proliferation induced by adrenaline (AD) and isoprenaline (ISO). Cells were pretreated with ICI for 45 min prior to incubation, and simultaneously with AD and ISO for 24 h. Cell proliferation was measured by BrdU incorporation assay, as described in Materials and Methods. *P<0.05, significantly different from the untreated control group. #P<0.01, significantly different from the respective concentration AD-treated group and ++P<0.01 significantly different from the respective concentration ISO-treated group.

shows that ATE significantly blocked AD- and ISO-induced cell proliferation, confirming the involvement of the β_1 subtype in the proliferative effect, through promotion of proliferation. ATE significantly decreased cell proliferation induced by AD at 1 and 10 μM to 55.7±5.6% (n=6) and 53.4±3.6% (n=6), respectively, and to 45.8±9.2% (n=6) and 32.1±4.6% (n=6) for ISO, respectively, at 1 and 10 μM. Furthermore, when applied alone, ATE decreased proliferation to 55.4±13.9% (n=6), compared to the control. To investigate the involvement of β_2-AR in HT-29 proliferation, the cells were incubated with ICI, a β_2-selective antagonist, either alone or with the AR agonists. As is evident in Fig. 8, ICI did not significantly affect

the proliferation induced by AD, whereas it reduced the effect induced by ISO 10 μM to 64.1±11.1% (n=7), reinforcing the involvement of the β_2-AR subtype in this process. ICI had no effect on HT-29 cell proliferation.

Discussion

Stress is considered to play a central role in the incidence and development of cancer (16). However, the molecular and cellular mechanisms by which stress increases the risk of certain types of cancer and their prognosis remain understudied. It has been postulated that endogenous CA mediate the association between stress response and poor cancer outcomes (10,17). Epidemiologic studies have associated the use of β-blockers in clinical settings to reduce the rates of progression for several solid tumors (18). Findings suggest that β-AR blockers may be inexpensive and safe therapeutic agents for cancer. The above studies mostly do not distinguish between β_1- or β_2-activity blockers, and the signaling pathways involved in these responses remain poorly understood.

In the present study, we addressed the effect of the stress hormones, AD and NA, and ISO, a synthetic β non-selective agonist, and several β-blockers, on colon cancer cell proliferation, a critical component of the carcinogenesis cascade. In tumoral cells, β-AR is the key receptor in mediating the effects of CA. The expression of β-AR has been identified in normal colon tissue and in colon cancer cells, including in HT-29 cells, β2-AR being the predominant receptor subtype in these cells (6). In fact, several studies have recognized the activation of β-AR as a central mediator of stress effects on cancer growth. The activation of these receptors is involved in different tumorigenic processes including proliferation (5), migration (4), apoptosis (19), angiogenesis (20) and differentiation in a various types of cancer (21). Therefore, β-AR blockade with pharmacological agents may be used to alleviate the effects of stress following cancer growth and progression. Concordant with this, findings of previous studies have shown that β-AR blockade may suppress cancer-cell invasion and inhibit adrenergic-driven metastasis (22).

In our study, the results obtained with the adrenergic agonists, confirmed that stress hormones affect colon-cancer cell proliferation, and suggest a prominent role for β-AR in this process. AD and ISO, as previously shown by other authors (5,6,23), significantly enhanced HT-29 cell proliferation, most likely through β-AR. These agonists, both with a high affinity for β-AR were strongly expressed in these cells, inducing a similar proliferative response in the present study. Based on the above observations, we explored the AR subtypes involved in AD and ISO effects, using β-blockers with distinct profiles for AR. Taken together, our results clearly indicate the involvement of the β-AR subtypes, β_1 and β_2, in promoting colon-cancer cell proliferation. PRO, the non-selective β-AR antagonist, was the most potent β-blocker in reversing AD effects following cell proliferation, by its ability to bind to the two subtypes, as identified in previous studies (23). The proliferation increase stimulated by AD was abolished by PRO, less by CAR, even less by ATE and was not affected by ICI. According to β_2 involvement, the β_1-blocker (ATE) was a weak antagonist for AD action, while the selective β_2-blocker (ICI) had no effect. By contrast, with the exception of ICI, which had a moderate effect, all the other β-blockers markedly inhibited the proliferation induced by ISO. Thus, unlike other reports (5,6,23), the β_1 blockade by ATE was more effective in reverting AD- and ISO-induced cell proliferation as compared to the β_2 antagonism by ICI.

As previously mentioned, β-blockers are not solely antagonists for the G-protein pathways, but they may independently modulate more than one pathway, and behave as partial agonists, inverse agonists or pure antagonists in each pathway, increasing the complexity of their actions (24). Thus, biased agonism may be important for the therapeutic use of β-AR blocker in cancer, since distinct signaling through these pathways is considered to have specific functional consequences (24). The β-AR blockers examined were already recognized as being inverse agonists (7). The finding that PRO and ATE, when used alone, were able to decrease HT-29 cell proliferation, suggests that they acted as inverse agonists, a function already described in other experimental models for the two drugs acting via β_1- and β_2-AR (7). The activation of β-ARs increases cAMP intracellular concentrations and promotes cell proliferation, two processes known to be reversed by treatment with either β_1-or β_2-AR antagonists (25). PRO and ATE acting as inverse agonists by binding to the β-ARs leads to a decrease of cAMP accumulation (7), an outcome that may explain the proliferation decrease induced by these drugs in our study. However, β_2 selective agonist (ICI) or CAR did not exert an antiproliferative effect when used alone, although both have been described as being able to decrease cAMP levels (26). Thus, as suggested previously (27), β-blockers seem to have complex profiles for cAMP modulation and Erk1/2 activation at β_1- and β_2-AR. Moreover, CAR is able to activate different signaling pathways depending on the cell type (26), although its effect on HT-29 proliferation was as yet unknown. CAR behaved similarly to PRO when used simultaneously with the two agonists. However, in contrast to data obtained with other cell types for β_1 and β_2, CAR had no effect as inverse agonist (26).

Ongoing investigation have focused on the downstream signaling pathways involved in β-AR-mediated tumor growth. Among the mammalian MAPK pathways, ERK is the one that is most studied, and the deregulation of this pathway occurs in approximately one-third of all human cancers (28). It has been previously concluded that β-AR-mediated ERK1/2 activation is a potential mechanism underlying stress-induced cancer cell growth *in vivo*, suggesting, for instance, that β-AR blockade may be an effective approach for patients with stress-related colon cancer (23). However, in 2006, Shenoy *et al* (30) showed in tumor models that ISO, by binding to β_2-AR leads to the activation of a G-protein-independent ERK pathway, although this was dependent on β-arrestin. The pathway whereby growth factors and mitogens activate ERK signaling is of particular relevance to cancer (28). The β-blockers used in our study, with the exception of ICI, have already been described as being capable of activating ERK pathway. However, as referred before, when used alone, ATE and PRO decreased cell proliferation and CAR had no effect.

CAR, PRO and ATE are widely used clinically. Several studies have shown that these and other β-blockers reveal new clinical applications in medicine, which is very attractive for commercial purposes. However, gaining understanding of β signaling pathways involved in cancer may allow the

identification and selection of the appropriate inhibitors to prevent and treat cancer. Corroborating the putative use of β-blockers as therapeutic agents in cancer, at least three phase II clinical studies assessing the safety and efficacy of β-blockers in breast, colorectal and ovarian cancers have been conducted (29).

Results of this study demonstrate the elucidation of the most effective β-AR blockers in reverting the CA-induced proliferative effects in colon cancer cells. Consequently, these blockers may be used as promising strategies in cancer treatment (in combination with other treatment paradigms).

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