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Artículo

Role of hydrogen peroxide prolonged treatment in LGR5 expression in colorectal cancer cells

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Abstract

Leucine-rich G-protein-coupled receptor 5 (LGR5) is a biomarker for cancer stem cells (CSCs), which activates the Wnt/β-catenin signaling pathway and promotes cell renewal and multiplication. Overexpression of this protein has been observed in several types of cancer, especially in colorectal cancer (CRC). In this study, we aimed to modulate mRNA and protein LGR5 expression in the mouse CRC cell line MC38 with a continuous and prolonged treatment with hydrogen peroxide (H2O2). Our real time PCR and western blot results revealed a significant increase of up to 20-fold in mRNA expression in H2O2-treated cells compared to non-treated cells after 27 days of treatment, with a slight increase in protein expression after 65 days of treatment with 75 µM of H2O2. However, in general decreased LGR5 protein levels were observed. Prolonged treatment with H2O2 at the doses tested in this study does not seem to be a good method to obtain MC38 cell line with LGR5 stable overexpression.

Keywords: Colorectal cancer, hydrogen peroxide, LGR5

Introduction

Colorectal cancer (CCR) is one of the most common types of cancer worldwide. Despite the wide range of existing treatments, they are not totally effective, and their side effects are very severe limiting the patient's quality of life. For this reason, the search for new therapeutic targets has become so important (Bray et al., 2021; Sung et al., 2021).

Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is a transmembrane receptor on the surface of stem cells located deep

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in the crypts of the intestinal epithelium (Fujii & Sato, 2017; Hagerling et al., 2020). It is a target protein of the Wnt/β-catenin signaling pathway and is activated by the binding to its ligand, R-spondin, resulting in enhanced cell proliferation and renewal (L. Yang et al., 2015), and therefore related to tumor progression. The malfunction of this pathway has been related to cancer progression (Katoh & Katoh, 2022; Zhang & Wang, 2020), so inhibitors of the Wnt/ β-catenin pathway have been developed as a therapeutic strategy (W. Yang et al., 2019).

LGR5 overexpression has been described in a large number of cancers, including colorectal cancer (CRC). High expression of this protein was correlated with increased tumor malignancy and decreased survival in patients (Gzil et al., 2020; Xu et al., 2019). Besides, it is overexpressed in cancer stem cells (CSCs), which are involved in the generation of metastasis and tumor relapses (Atashzar et al., 2020; Paolillo et al., 2019). There are studies that highlight the enormous plasticity in the expression level of this protein, with the clinical implications that it could have as a clinical target (Morgan et al., 2018). LGR5 expression may be modulated by several mechanisms that involve the Wnt/β-catenin pathway. Specifically, inhibition of the MEK1/2 protein (RAS signaling pathway), increased LGR5 expression (Zhan et al., 2019), inhibition of the furin protein, and decreased LGR5 expression in KRAS or BRAF mutated colon tumors (Descarpentrie et al., 2022). Furthermore, oxidative stress may stimulate the Wnt/β-catenin pathway, promoting the activation of β-Catenin and increasing cytoplasmic APC (Catalano et al., 2021), and therefore enhancing LGR5 gene and protein expression after 24 hours of treatment (Kim et al., 2012). Thus, the aim of this study was to modulate LGR5 expression in a mouse CRC cell line by the generation of oxidative stress with the prolonged treatment with H2O2 to enhance LGR5 expression (LGR5+++).

Materials and methods

Cell culture

The mouse colon cancer cell line MC38 was kindly provided by Dr. J. Scholl (Public Health Service, National Institutes of Health, Bethesda, MD, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture (Sigma-Aldrich, Madrid, Spain) in an environment with 5% CO2 at a constant temperature of 37ºC. This environment is controlled to ensure cell survival and growth under optimal conditions.

Cell proliferation assay

Cells were seeded at a density of 3x103 cells/well in a 48-well plate and incubated overnight. Then, MC38 cells were exposed to increasing concentrations of H2O2 (Sigma-Aldrich) from 10 to 100 µM and cell viability was measured at 24, 48 and 72 hours by the modified protocol of sulforhodamine B (SRB) colorimetric assay (Ortiz et al., 2015). Briefly, cells were fixed with 250 µl trichloroacetic acid (TCA; 10%) for 20 min at 4°C and washed with distilled water three times. Then, the samples were stained with 250 µl sulforhodamine B (SRB; 0.4%) for 20 min under stirring. After three washed with 1% acetic acid,

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SRB was resuspended with, Trizma® 10 mM, pH 10.5 and measured spectrophotometrically at 492 nm (Titertekmultiscan Colorimeter, Flow, Irvine CA, USA). Finally, the proliferation value relative to the untreated negative control was calculated with the following formula: Relative expression (%) = $\frac{\text{Absorbance of the sample}}{\text{Absorbance of the negative control}} \times 100$

LGR5 expression modulation through H_2O_2 treatment

Based on proliferation assay results, non-toxic concentrations of H_2O_2 were chosen for prolonged treatment of MC38 cells. During the treatment, the culture medium with H_2O_2 was renewed every 2-3 days until 65 days. Non-treated cells, and cells treated 27 and 65 days with H_2O_2 were collected and processed for RNA and protein expression levels analysis (see below). In addition, light microscopy images (CKX541 inverted microscope, Olympus) were taken to detect changes in cell morphology along the treatment.

Real time qPCR

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The RNeasy Mini kit (Qiagen, MD, USA) was used to extract total RNA from the samples following the manufacturer's instructions. Then, RNA concentration was measured using NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA) and complementary DNA was obtained (cDNA) using a retrotranscriptase kit (Promega, Madison, WI, USA), following the manufacturer's instructions. SYBR green supermix (Taq Universal SYBR Green Supermix) (Bio-Rad Laboratories, Hercules, CA, USA) and the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) were used for real-time PCR. The number of qPCR cycles was 45 and the annealing temperature used was 60ºC. The qPCR primers used for mouse LGR5 gene are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control and the results were expressed as 2(-ΔΔCt).

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Table 1. Primers used for qPCR experiment.

Gene		Primer	Tm°
mLGR5	Forward	5'-AGAACACTGACTTTGAATGG-3'	56,7
	Reverse	5'-CACTTGGAGATTAGGTAACTG-3'	55,5
mGAPDH	Forward	5'-CTAATGACCACAGTCCATTC- 3'	56,9
	Reverse	5'-GATGGGATGATGTTTTGGTG-3'	62,7

Western blot

The proteins were obtained from the samples with RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) and their concentration was measured using the Bradford method (Sigma-Aldrich, Madrid, Spain). A total of 40 µg of proteins were heated at 65°C for 20 min and separated on an 8% SDS-PAGE. Then, proteins were transferred to a 45 µm pore size nitrocellulose membrane (Millipore, Burlington, MA, USA) and blocked with a 5% milk solution in TBS with 0.1% Tween-20 (Bio-Rad, Hercules, CA, USA) for one hour. After that, they were incubated with anti-LGR5 primary antibody (1:500) (Anti-LGR5 (634J2E): sc-51766; Santa Cruz Biotechnology) overnight at 4°C, followed by secondary antibody (1:20000) (m-IgGκBP-HRP: sc-516102) for one hour at room temperature. β-actin was used as an endogenous control (1:25000 dilution) (Monoclonal Anti-β-Actin-Peroxidase antibody produced in mouse A3854; Sigma-Aldrich). The data obtained were analyzed with the open source software imageJ.

Results and discussion

Cell proliferation assay

The viability of the MC38 cell line was evaluated at increasing concentrations of H2O2. The maximum non-toxic concentration obtained was 75 µM in all exposition times (Figure 1A). From this point, a decrease in cell viability was observed, which can even be reduced to below 50% at 48 and 72 hours of exposition. Finally, the doses of H2O2 selected for the prolonged treatment were 60, 75, and 85 µM. After 27 days of H2O2 treatment significant differences in cell morphology and growth capacity were observed compared to the non-treated cells, such as a more elongated cell shape and a higher amount of dead cells in the culture medium (Figure 1B). This is due to the nature of peroxide as a reactive oxygen species (ROS), which decreases normal cell growth mainly by causing damage to cell membranes (Magder, 2006). Oxidative stress, interestingly, has contradictory effects on cancer progression. Cancer cells generate a lot of ROS because of their high metabolic activity, and in consequence have increased antioxidant mechanisms (Azmanova & Pitto-Barry, 2022). However, despite the inherent

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toxicity, it has been previously described that moderate concentrations of ROS can participate in signaling pathways, modulating the expression of certain proteins (Kunsch & Medford, 1999; Roux et al., 2019).

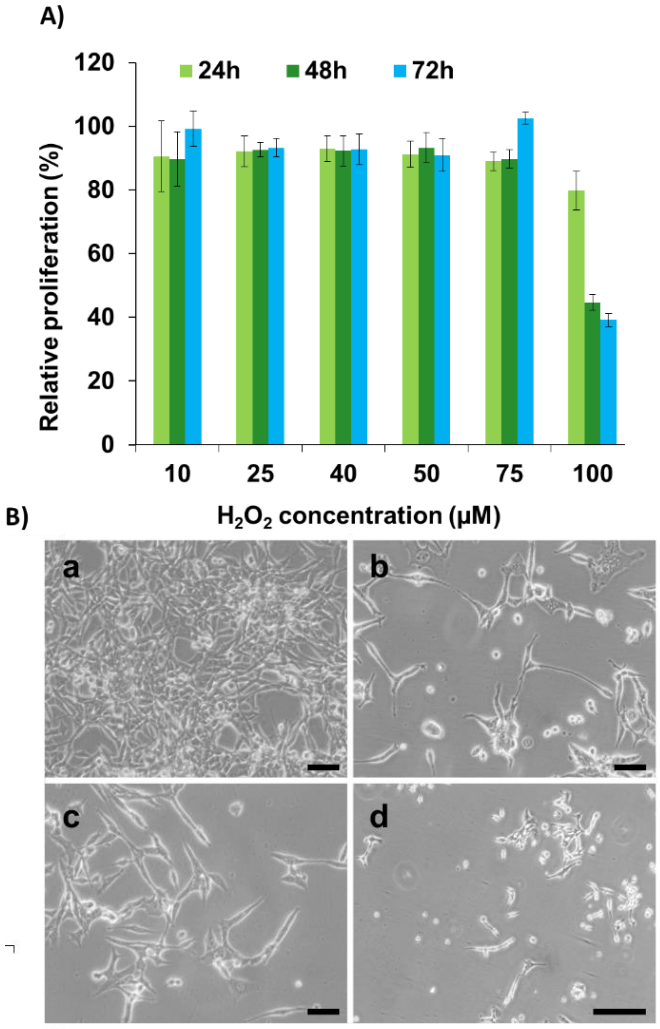

Figure 1. Cell viability after H2O2 short treatment. (A) Relative cell proliferation of MC38 mouse CRC cell line treated at increasing doses of H2O2 for 24, 48 and 72 hours. (B) Light microscopy images of (a) non-treated MC38 cell line, and treated with (b) 60, (c) 75, and (d) 85 µM of H2O2 (magnification (a, b, c) 20x and (d) 10x). Data have been expressed as the mean of triplicate cultures ± standard deviation.

LGR5 expression analysis through qPCR

RNA was extracted from all the samples and RT-PCR followed by qPCR was performed. The results obtained showed a 9.66- and 20-fold increase in LGR5 mRNA expression in cells treated with 75 and 85 µM H2O2 for 27 days, respectively, compared to untreated cells (Figure 2). After 65 days of treatment a slight overexpression of LGR5 mRNA was maintained in the 60 µM treatment (2.67-fold) (Figure 2). The rest of the samples did not show significant differences compared to the negative control. Therefore, until at least 27 days there is an overexpression of LGR5 mRNA induced by hydrogen peroxide treatment, but prolonged H2O2 (65 days) treatment cannot achieve these increases in mRNA LGR5 expression, obtaining lesser overexpression values. The effectiveness of hydrogen peroxide-based treatment has also been analyzed at shorter times (24 hours), obtaining favourable results at higher concentration (50-300 µM) (Kim et al., 2012). Hence, it is possible that a H2O2 treatment may not be stable over long periods of time, mainly because of its instability, as its effect on cells may vary due to the decomposition of the compound under certain conditions, which complicates the analysis of results (Schmidt et al., 2011).

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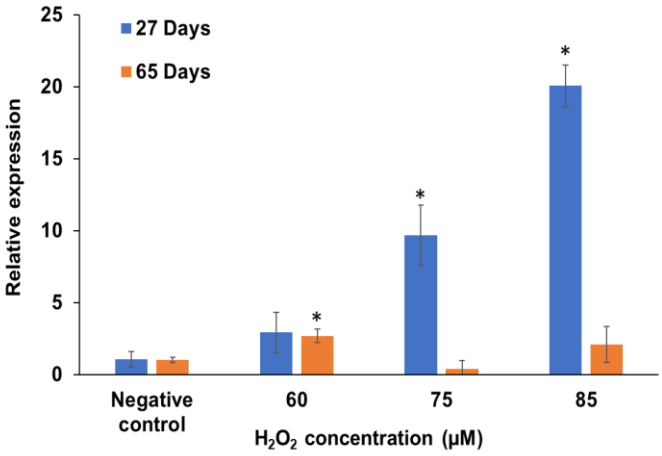

Figure 2. Relative LGR5 mRNA expression of the murine CRC MC38 cell line untreated and treated with 60, 75 and 85 µM of H2O2, after 27 and 65 days of treatment. GAPDH gene was used as endogenous control and the results were expressed as the 2(-ΔΔCt). Data have been expressed as the mean (n=3) ± standard deviation. (*) Statistically significant differences with respect negative control ($p < 0.05$).

LGR5 protein expression analysis by western blot

There was no increase in LGR5 protein expression after 27 days of treatment and a slight increase after 65 days (Figure 3) despite the expression values obtained in mRNA probably due to alternative splicing events and post-transcriptional changes (Ule & Blencowe, 2019; Wegler et al., 2020).

Moreover, the generation of oxidative stress in cancer cells may activate the Wnt/β-catenin pathway (Catalano et al., 2021), LGR5 is a product of the Wnt/β-catenin pathway and a positive regulator (Carmon et al., 2012), but could have a bi-modal regulation, as an activator with the normal activity of the Wnt/β-catenin pathway, and as an inhibitor when this signaling pathway is unusually overexpressed (Fernandez Vallone et al., 2020; Walker et al., 2011; Wu et al., 2014). Therefore, prolonged treatment with H2O2 could have enhanced the Wnt/β-catenin pathway in a way that has produced an effect contrary to the expected by the inhibition of LGR5 protein expression by the negative feedback of the pathway. In addition, we observed that higher concentrations, such as 85 µM at prolonged time treatment (65 days) decreased LGR5 protein levels. This could be related to the negative feedback of the Wnt/β-catenin pathway but also because of the excess of H2O2 that could activate cell apoptosis mechanisms. This was observed by Kim et al. at higher doses (600 µM) and short exposure times (1 day), showing that excess H2O2 can have negative effects on cell viability (Kim et al., 2012).

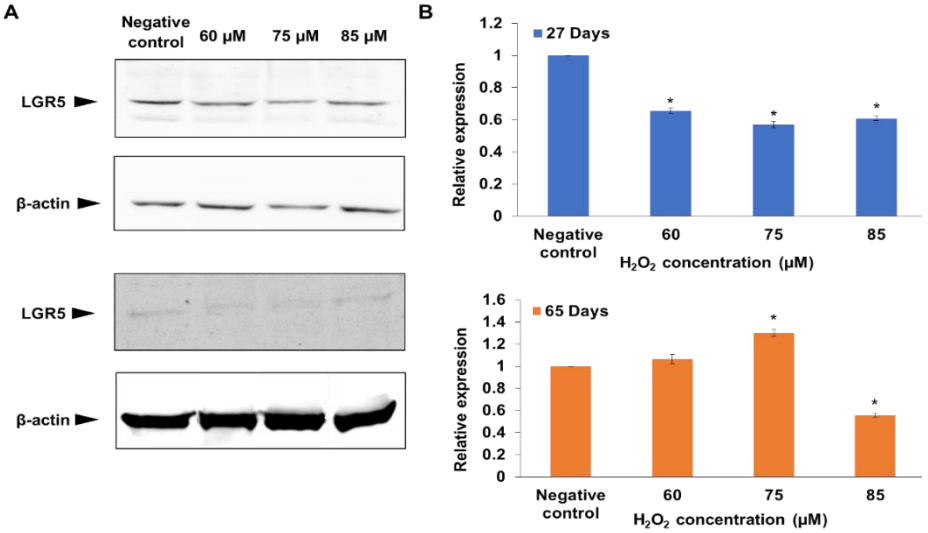

Figure 3. LGR5 protein expression assay. (A) Western hybridization images and (B) relative protein expression at 27 and 65 days (up and down respectively) of LGR5 protein expression in MC38 cells with 60, 75 and 85 µM H_2O_2. β-actin was used as endogenous control. (*) Statistically significant differences with respect negative control ($p < 0.05$).

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Conclusion

We demonstrated that LGR5 mRNA expression appears to be modulated in the MC38 cell line after 27 days of treatment with H2O2, but slightly increased expression was only observed with the 75 µM treatment after 65 days. Moreover, high concentrations of H2O2 (85 µM) at prolonged time treatment (65 days) decreased LGR5 protein levels. Prolonged treatment with H2O2 could enhance the Wnt/β-catenin pathway with a consequent decrease in LGR5 protein expression by the negative feedback of the pathway due to the bi-modal regulation by LGR5. Therefore, the treatment with H2O2 at the doses tested in this study does not seem to be a good method to obtain a mouse CRC cell line with a stable overexpression of LGR5.

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