AMERICAN UNIVERSITY OF BEIRUT

DIFFERENTIAL EXPRESSION OF BRADYKININ AND RETINOIC ACID RECEPTORS IN HUMAN COLORECTAL CANCER CELL LINES

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biochemistry and Molecular Genetics
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
August 2015

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ACKNOWLEDGEMENTS

First, I would like to thank my thesis advisor Dr. Ayad Jaffa, Chair and Professor of the Biochemistry and Molecular Genetics Department at AUB, for allowing me to join his laboratory to conduct the experiments needed to complete the requirements for a Masters of Science in Biochemistry. Dr. Jaffa encouraged me to become an independent scientist, to troubleshoot failures and acquire an analytical spirit. I would also like to thank the committee members, Dr. Ayad Jaffa, Dr. Hiba El Hajj, Dr. Nadine Darwiche and Dr. Fuad Ziyadeh, for reviewing my thesis and their technical and educational support in my master's study.

My recognition and gratitude are addressed to the 2-12 laboratory members: Lamis Saad, Moustafa Al Hariri, Joseph Abou Msallem, Oula Dagher, Rana Ghali, Nahed Mogharbel, Ruwaydah Kabbani, Sally El Sitt, Patrick Zakka, and Rana Abdel Samad and Zeinab Sweidan in Dr. Darwiche's laboratory, for teaching me all that I need regarding techniques and experiments, and more importantly, for being my family outside home. I really can't thank you all enough for all your support throughout my journey.

My appreciation and gratitude to all my professors here at AUB for encouraging critical thinking, looking for evidence as opposed to taking things as they are, through their teaching methods. I really enjoyed all classes I took during my degree and learnt a lot of interesting material which will definitely help and guide me throughout my carrier. It was a true honor to sit in your classes. Thank you.

None of this, of course, would have been possible if it was not for my parents, Fouad and Danielle, and my siblings, Paula and Antoine to whom I dedicate this work. My family was there at all steps encouraging me, supporting me in all means and being my inspiration to be able to fully accomplish my work.

I would like to express my special thanks to my beloved and supportive fiancé Marc, who was and always is by my side, supporting me in every single step I make.

Above all, greatest appreciation to the Almighty God who has given me the health and strength to finish this work.

Thanks again to everyone.

AN ABSTRACT OF THE THESIS OF

Celine Fouad Khati for Master of Science
Major: Biochemistry

Title: Differential Expression of the Bradykinin and Retinoic Acid Receptors in Human Colorectal Cancer Cell Lines

Chronic inflammation is a major characteristic of the development and progression of several tumors, in particular colorectal cancer. The connection between inflammation and tumorigenesis has been well supported from pharmacological, epidemiological, and genetic data. However, the molecular mechanism by which inflammation promotes cancer cell growth constitutes a broad area of ongoing investigation. Hence, multiple key genes and signaling pathways involved in oxidative stress and inflammation, such as the kallikrein-kinin system, may play a key role in tumorigenesis. In fact, bradykinin has been shown to modulate tumor progression. We have also shown that bradykinin activates the extracellular signaling regulated kinase (ERK) 1/2 pathway which may promotes cell survival or cell death in cancer cells. Retinoids are major regulators of epithelial cell proliferation, apoptosis, and differentiation and have therefore been used in the prevention and treatment of some cancers. Retinoids have also shown promise in preclinical colorectal cancer studies. Therefore, we were interested in investigating a potential crosstalk between the kallikrein-kinin system, and the retinoic acid signaling pathway using a well-characterized human *in vitro* colorectal cancer model.

In the present study, we tested for the effects of bradykinin receptor 1 and 2 (B1R and B2R) agonists on the proliferation of human colorectal cancer cells, and on the expression and modulation of B1R, B2R, and retinoid receptors (RARα, RARγ, RXRα). We investigated as well the subcellular localization of RARγ, and B2R upon treatment with its high affinity ligand bradykinin, and some of the downstream effector signaling pathways.

We showed by MTT assay that the growth of the colorectal cancer cell lines (HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$) was not affected by B1R and B2R agonists. However, we observed by real-time PCR and western blot analysis a differential expression of B1R, B2R, and retinoid receptors transcripts and proteins upon B1R and B2R ligand binding. Interestingly, bradykinin treatment of the different colorectal cancer cells resulted in inhibition of Protein Kinase B phosphorylation, and induction of ERK1/2 phosphorylation and in a spatiotemporal crosstalk between RARγ and B2R in the subcellular compartments.

Future studies will investigate the potential relationship between bradykinin and retinoic acid signaling pathways, to test whether their activation or inhibition regulates inflammation, cell proliferation, and cell death. These studies may provide therapeutic opportunities for the treatment of colorectal cancer.

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ABBREVIATIONS

ATRA	all-*trans* retinoic acid
AKT	protein kinase B
9c-RA	9-*cis* retinoic acid
B1R	bradykinin 1 receptor
B2R	bradykinin 2 receptor
BSA	bovine serum albumin
Ca^{++}	calcium
CDK	cyclin-dependent kinase
CO_2	carbon dioxide
DAPI	4,6-diamidino-2-phenylindole
cDNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
EGFR	epidermal growth factor receptor
ERK 1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
IL	interleukin
μM	micromolar
μL	microliter
μG	microgram
MAPK	mitogen-activated protein kinase
MMPs	matrix metalloproteinases
NADH	nicotinamide adenine dinucleotide

NADPH	nicotinamide adenine dinucleotide phosphate
ND	nanodrop
NF-kB	nuclear
OD	optical density
PBS	phosphate buffered saline
RAR	retinoic acid receptor
RA	retinoic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-qPCR	real time-quantitative polymerase chain reaction
RXR	retinoid x receptor
SDS-PAGE	sodium dodecyl sulfate-polyacralamide gel electrophoresis
SEM	standard error of the mean
ST1926	E-4-(4’-hydroxy-3’-adamantyl biphenyl-4-yl) acrylic acid
TBST	tris buffer saline tween
TGβ 1 and 2	transforming growth factor 1 and 2
VEGF	vascular endothelial growth factor

CHAPTER I

INTRODUCTION

A. Colorectal Cancer Overview

1. Prevalence and Treatment of Colorectal Cancer

Cases of colorectal cancer are continuously increasing worldwide and having more than one million new cases diagnosed each year. Colorectal cancer has been classified as the third most common malignancy and cause of cancer death in the world. It is equally manifested in men and women, with a higher risk of incidence starting above the age of fifty (Siegel, Miller, & Jemal, 2015). A small percentage of colorectal cancer patients are considered having a heritable genetic basis (with 5% accounted for hereditary non-polyposis colon cancer and familial adenomatous polyposis), whereas the biggest fraction of colorectal cancer cases is mainly linked to intestinal polyp progression and environmental causes (Tenesa & Dunlop, 2009). Risk factors include food-borne mutagens, specific intestinal pathogens (Dahm et al., 2010), adopting a high fat diet (Marshall, 2008), being diabetic or overweight (Potter, 1996), heavy consumption of alcohol and caffeine, smoking and chronic intestinal inflammation such as an inflammatory bowel disease (Crohn's disease/ulcerative colitis), which usually precedes tumor development and progression (Cho, Lee, Rimm, Fuchs, & Giovannucci, 2012). Unfortunately, a high percentage of cases are detected at an advanced stage whereby poor prognosis is established. This is due to the fact that most people do not show any early signs or symptoms of the disease. Advanced stages of colorectal cancer are known to be manifested by blood in stools, abdominal discomfort, unexpected weight loss, pain upon bowel movement and fatigue among others (Saratzis, Winter-Beatty, El-Sayed, Pande, & Harmston, 2015).

For that reason, performing regular screening tests such as colonoscopy, especially at an age of fifty, is highly advisable and considered crucial for an effective preventive method of colorectal cancer (Stock, Knudsen, Lansdorp-Vogelaar, Haug, & Brenner, 2011). Moreover, colonoscopies are able to detect polyps before they can become malignant, and thus preventing and reducing up to 19% of colorectal cancer deaths. When detected at an early stage, the five-year relative survival rate is 90%, yet only around 40% of colorectal cancer cases are diagnosed at this stage. This is primarily attributed for the underuse and/or underestimation of the importance of screening tests (John et al., 2014).

Treatment of colorectal cancer can be aimed at cure or palliation. It varies according to the tumor location, stage and whether and where it has spread. Surgery to remove the cancer and surrounding lymph nodes if need be, is the most common treatment which includes polypectomy, colectomy, proctectomy, and proctocolectomy. This is followed, in most cases, by a combination of an average of six months chemotherapy with various targeted drugs to lower the risk of recurrence and metastasis (Ahmed, Johnson, Ahmed, & Iqbal, 2014). One of the newest and clinically used drug is the 5-Fluorouracil which was proven effective in the treatment of colorectal cancer. As for radiation therapy, it is not recommended for colon cancer since bowels are relatively sensitive to radiation. Recurrence, within the first three years after surgery, is not an uncommon feature among colorectal cancer survivors, as a secondary tumor is most likely to develop in other organs of the digestive system mainly the liver (Bonithon-Kopp, Kronborg, Giacosa, Rath, & Faivre, 2000). Metastasis, leads to a drop of 70% in the five-year survival rate.

Colorectal cancer is considered a highly treatable disease only if it is caught early in time. Unfortunately, the symptoms of this type of cancer can be puzzled with other pathological

conditions like infections, hemorrhoids, among others. Despite all advances in the chemotherapy and surgical fields in order to cure colorectal cancer, these treatments can be aggressive with a relatively low cure index (Azcarate-Peril, Sikes, & Bruno-Barcena, 2011). Prevention, early diagnosis, regular screening tests with specific biomarkers, in parallel with adopting a healthy lifestyle, remain the best ways to decrease the risk factors, impede the progression and encumber the malignant manifestations of colorectal cancer.

2. Genetic and Epigenetic Alterations in Colorectal Cancer

Normally, growth of human cells is controlled by many genes which regulate various cell processes such as proliferation, cell death, and differentiation (Hanahan & Weinberg, 2011). However, in a cancer state, alterations in these genes usually occur one after the other (Gaude & McCormick, 1999). Moreover, the first mutation that occurs will eventually offer a selective growth advantage to the cell. Thus, the developing tumor will acquire at that stage mutations in oncogenes, tumor suppressors or stability genes, which lead to an ongoing cellular division and mass of cells that will ultimately form the primary tumor (Da Pozzo et al., 2007). These genetic mutations could be inherited, or can arise with time. On the other hand, disruption of epigenetic processes leads to a deregulated gene expression which results in cancer development (Figure 1). Examples of epigenetic deregulations include DNA methylation, histone modifications, chromatin structure, and non-coding RNAs (Schneider-Stock, Ghantous, Bajbouj, Saikali, & Darwiche, 2012).

B. Kallikrein-Kinin System

The kallikrein-kinin system is known to be involved in vascular permeability, coagulation, pain and inflammatory processes following tissue injury (Calixto, Cabrini, Ferreira, & Campos, 2000) (P. H. Wang et al., 2009). The kallikrein–kinin system consists of two serine proteases, plasma and tissue kallikrein, that enzymatically cleave hepatically-derived kininogens and release bioactive kinin peptides known as bradykinin and Kallidin. The term "kinin" refers to the nonapeptide namely bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), the decapeptide kallidin (KD: Lys-BK), the methionyl-lysyl-BK, and their carboxy-terminal des-Arg metabolites. Kinins are released from molecules called kininogens (Figure2) (da Costa, Sirois, Tannock, & Chammas, 2014). This can be triggered by a wide range of physiological and pathological events (Bozo, Eles, & Keseru, 2012) (Couture, Harrisson, Vianna, & Cloutier, 2001). Kininogens are multifunctional proteins which are major players in the inflammatory cascades, and were recently showing more involvement in carcinogenesis. They are among the most potent pro-inflammatory vasoactive peptides generated during noxious stimulation or injury (Ehrenfeld, Figueroa, Matus, & Bhoola, 2012).

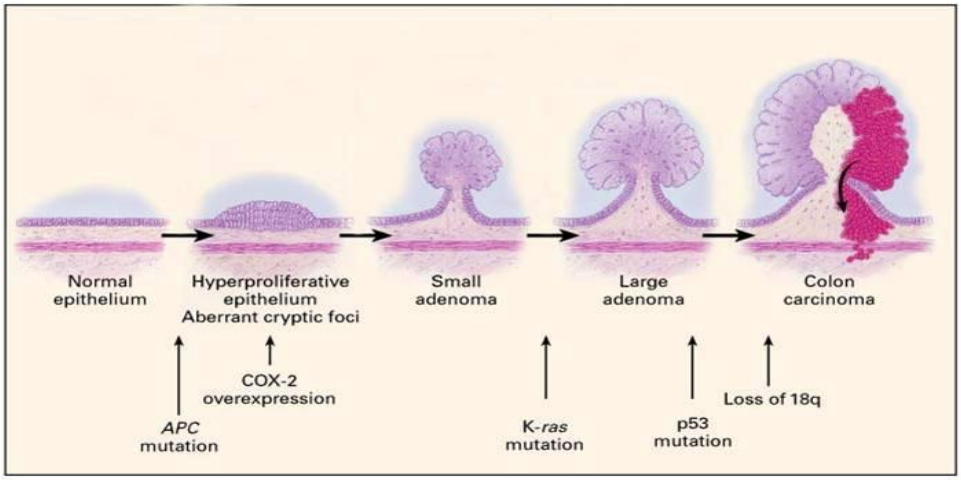

Figure 1. An Inducible Mouse Model of Colon Carcinogenesis for the Analysis of Sporadic and Inflammation-Driven Tumor Progression. Modified and adopted from (Becker 2007).

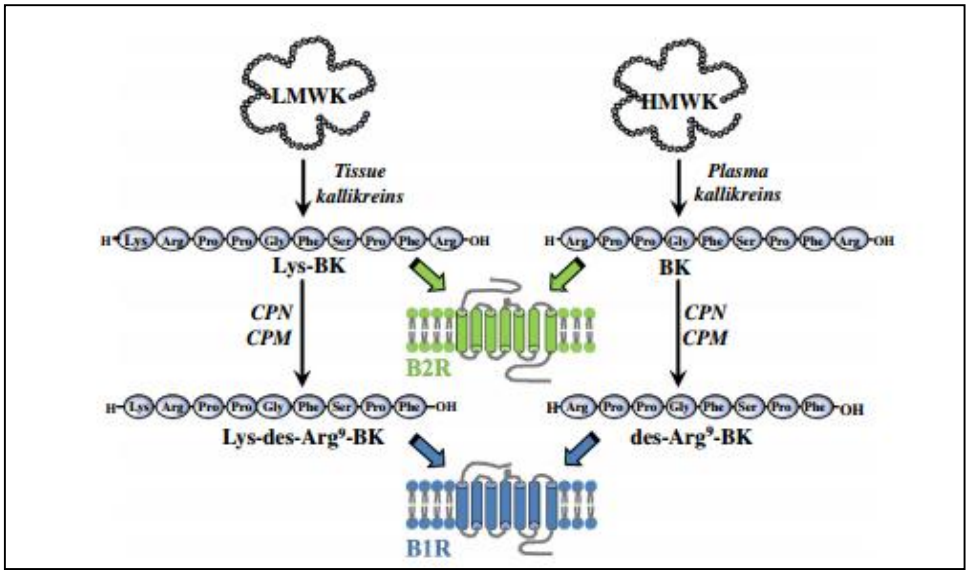

Figure 2. Schematic representation of the kallikrein–kinin system. The kininogens LMWK and HMWK are cleaved by tissue and plasma kallikreins, respectively, generating the metabolites BK and Lys-BK, respectively, which can be cleaved by the kininases CPN and CPM generating respectively the metabolites des-Arg^9-BK and Lys-des-Arg^9-BK. Lys-BK and BK are agonists of B2R and Lys-des-Arg^9-BK and des-Arg^9-BK are agonists of B1R. Modified and Adopted from (da Costa 2014).

C. Bradykinin and Cancer

It has been reported that bradykinin plays an important role in the progression of many types of cancer. Many solid tumors are found to be surrounded by an inflammation zone evoked by bradykinin (Maeda, Wu, Sawa, Matsumura, & Hori, 2000). For example, bradykinin is thought to be involved in colorectal cancer cell invasion and migration. The mechanism through which bradykinin works may be related to the overexpression of Bradykinin 1 Receptor (B1R) and Bradykinin 2 Receptor (B2R), in the colorectal cancer cells along with the stimulation of extracellular signal-regulated kinases 1/2 (ERK1/2) activation and interleukin-6 (IL-6) production (Stewart, 2003).

B1R and B2R could play an important role in tumor growth, development and metastasis since they are mainly implicated in inflammatory pathways and mitogenesis. The administration

of bradykinin in human and animal tissues led to the development of the four cardinal signs of inflammation: redness, local heat, swelling, and pain (Gomis et al., 2013). The biological behavior of tumors is in part due to the ability of kinins to induce cell proliferation, migration, vascular permeability, and angiogenesis. Vascular permeability facilitates the expansion and migration of cancerous cells as well as angiogenesis has been shown to be highly related to inflammation and more importantly induced by bradykinin (Golias, Charalabopoulos, Stagikas, Charalabopoulos, & Batistatou, 2007).

Several cancers including renal, esophageal, cervical, gastric, prostate, lung and mammary carcinomas, malignant mesothelioma and cancer cell lines, demonstrated a relatively high expression of B1R. Furthermore, B2R was detected in head and neck squamous cell carcinoma, osteosarcoma, endometrial, prostate, renal, cervical, lung and stomach cancers, hepatoma, lymphoma, mesothelioma, and pituitary adenoma progression (da Costa et al., 2014). Studies that involved the two receptors' expression levels in clinical samples from cancer patients and cancer cell lines have suggested a potential role in malignant transformation and tumor progression (da Costa et al., 2014).

Based on the latter, bradykinin antagonists could be an interesting option for the treatment of tumors because of their anti-inflammatory and anti-angiogenic properties (J. Wu, Akaike, & Maeda, 1998). In addition, some types of tumors produce bradykinin and use it in an autocrine fashion to stimulate further their own growth namely in small cell carcinoma of lung, prostate cancer, and certain ascites tumors. Bradykinin antagonists have shown some promising results as anti-cancer drugs without evident toxic effects on the host animals which is unlikely with present chemotherapeutic drugs used in clinics for the treatment of tumors (Morbidelli et al., 1998). In fact, several B1R and B2R antagonists were tested in clinical trials of many

diseases including cancer. So far, only the B2R antagonist HOE-140 was approved for hereditary angioedema and was tested for other conditions (da Costa et al., 2014).

Several studies have shown that the activation of kinin receptors leads to the activation of the ERK 1/2 pathway, epidermal growth factor receptor (EGFR), and production of matrix metalloproteinases (MMPs) in tumor cells. In *in vitro* models, B1R and B2R were found to induce the accumulation of MMPs and thus contributing to the invasiveness properties of cancer cells. Moreover, bradykinin induces MMP-9 expression *via* reactive oxygen species (ROS) dependent pathways in brain astrocytes (C. C. Lin et al., 2012).

There is an urge for discovering and identifying clinically useful biomarkers for detection of cancer at its early stage. Only a few examples of them can be used in detecting cancers before they are clinically evident. For example, 3-hydroxyprolyl (^{3}Hyp)-bradykinin is abundant in the ascitic fluid of gastric cancer patients. Recently, bradykinin and B1R agonist levels were found to be higher in breast than bladder cancer patients when compared to normal subjects and that after removal of the tumor their level was reduced. Further studies should be done to evaluate and confirm the potential of bradykinin, in particular the B1R agonist, as biomarkers in the diagnosis and therapeutic management of cancer patients (da Costa et al., 2014).

Therefore, based on the previous studies, bradykinin may bear an autocrine/paracrine mechanism through which it promotes the effect of kinins, leading to signal amplification and tumor growth, vascular permeability, angiogenesis and MMPs activation (Figure 3). It is noteworthy to emphasize that tumors are heterogeneous and do not only consist of neoplastic host cells but also inflammatory cells such as endothelial cells, leukocytes, and fibroblasts (Hanahan & Weinberg, 2000). The different cell types in the tumor and its microenvironment express kinin receptors and may be crucial players in tumor progression. Many malignancies

arise from chronic infections, suggesting that cancer may develop from areas of inflammation as part of the normal physiological response of the host. Furthermore, studies have supported the idea that host cells might lead to an increase in B2R expression level, which in turn facilitates tumor-associated angiogenesis by up-regulating vascular endothelial growth factor (VEGF) production and this mainly in fibroblasts (Ikeda et al., 2004).

The activity of nuclear retinoid receptors in particular the retinoic acid receptors (RARs) can be controlled by signals transduced by membrane-associated receptors such as the B1R and B2R, and this could be through the growth factor/Ras/Raf/mitogen-activated protein kinase (MAPK) cascade. This makes RARs effective molecular switches that are able not only to transduce retinoid signals but also some other signals received by the membrane-associated receptors (Tazzari et al., 2014).

Based on the above, a potential crosstalk between bradykinin receptors and RARs signaling pathways should be elucidated, leading to a better understanding of the molecular pathways joining kinins and retinoids. The ultimate goal is to develop target drugs for the treatment of life-threatening diseases such as cancer and more specifically colorectal cancer.

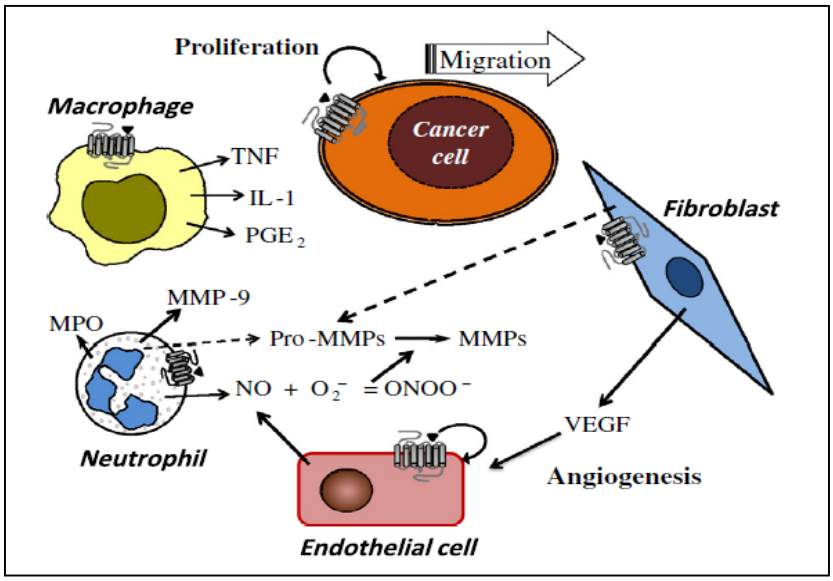

Figure 3. Schematic drawing of some mechanisms involved on the activation of kinin receptors within tumor microenvironment. It has been suggested, according with *in vitro* and *in vivo* studies, that kinin receptors activation in different cells that compose the tumor microenvironment results in a range of actions, such as the release of MMPs, growth factors and inflammatory mediators, which can promote tumor growth, angiogenesis, invasion and cancer metastases. Adopted from (da Costa 2014).

D. Kinin Receptors in Cancer

B1R and B2R belong to the G-protein coupled receptor family, which mediate kinins effects through the activation of a number of signaling molecules, such as, several isoforms of protein kinase C and phospholipases and the generation of second messengers, such as inositol-1,4,5-trisphosphate, diacylglycerol, calcium (Ca^{++}), and arachidonic acid which is subsequently converted to prostaglandins. These two kinin receptors are located on the plasma membranes of many cell types including endothelial, epithelial, neural, smooth muscle cells, neutrophils, lymphocytes, monocytes, keratinocytes, chondrocytes , and fibroblasts (Greco et al., 2005) (Bawolak, Gera, Morissette, Stewart, & Marceau, 2007) (Ikeda et al., 2004). Depending on the cell type, and upon ligand stimulation, B1R and B2R activate diverse intracellular pathways

which regulate cell proliferation, differentiation, migration, vascular permeability, excitation of nerve endings, and contraction of smooth muscle cells in addition to the release of multiple secondary mediators involved in various biological processes (Ehrenfeld et al., 2012).

Previous studies have shown that B1R is expressed to low levels in healthy tissues; however, proinflammatory cytokines or growth factors enhance its expression during tissue injury, inflammation, cancer, and exposure to bacterial endotoxins. In addition, an up-regulation of this receptor was observed in several tumors, immune-modulated disorders, transplant rejection, glomerulonephritis, and human fibrotic lung tissue (S. Li, Huang, & Peng, 2005). Some studies have reported crosstalk between B1R and B2R with evidence that persistent stimulation of the B2R may result in up-regulation of the B1R (Barki-Harrington et al., 2003). Further evidence demonstrated that B1R heterodimerizes with bradykinin receptor 2 and other molecules as well as mediates the actions of B1R agonist, a carboxyterminal truncated metabolite of bradykinin (Taub, Guo, Leeb-Lundberg, Madden, & Daaka, 2003). B1R activates the majority of the signaling pathways activated by B2R, and the antagonism of one receptor was seen to interfere with the ability of the other (Prinster, Hague, & Hall, 2005). B1R activity is controlled by multiple signaling pathways such as stress mitogen-activated protein and nuclear factor kappa B (NF-kB). This further potentiates the signaling of bradykinin receptor 1 and uncovers its molecular implication in various intracellular pathways (Ehrenfeld et al., 2012).

Although these two receptor's activation triggers essentially the same signaling pathways, the patterns of signaling are different in duration and in intensity of cell Ca^{++} influx (Table 1). B2R is internalized and recycled to the cell surface by its agonist, and is stable even in the absence of its agonist but undergoes a rapid desensitization by a mechanism known to involve β-arrestins. B2R signaling is transient whereas B1R signaling is known to be sustained.

The latter is constitutively internalized in the absence of its agonist. Upon agonist binding, B1R internalization is inhibited leading to a delayed degradation of this receptor (Enquist, Skroder, Whistler, & Leeb-Lundberg, 2007).

It is worth mentioning again that the B1R involvement in different pathways is related to the cell type involved. Studies have shown that in primary cultures of arterial smooth muscle cells, B1R inhibited cell migration, whereas in PC3 prostate cancer cells, it favored cell migration through the activation of a crucial kinase involved in cytoskeletal reorganizartion and cell migration namely the focal adhesion kinase (Lu, Leung, Huang, & Wong, 2010). Increased levels of B1R were expressed in colorectal adenomas which tend to progress to malignancy whereas increased levels of B2R were found in hyperplastic polyps with low neoplastic potential (Zelanski & Fisher, 2006). However, little is known on the effect of the above mentioned receptors on colorectal cancer cells and tumor progression.

Bradykinin 1 Receptor (B1R)	Bradykinin 2 Receptor (B2R)
Sustained signal	Transient signal
Inducible	Constitutively expressed
Both receptors can be expressed by the same cell type	

Table 1. The difference between B1R and B2R.

E. Vitamin A and Retinoids

1. Overview

The first report involving vitamin A was done by Magendie in 1817 (Lanska, 2009) and its effect was first described in a mouse experiment by G. Lunin in 1881 (Voss, 1956). This essential component was called "fat-soluble factor A" after it has been tested and well-studied by McCollum in 1907 for its ability to restore health and support growth and development in rats (Lanska, 2009).

Retinoids are natural vitamin A derivatives or synthetic analogues with vitamin A activities. There are over four thousands natural and synthetic molecules considered related to vitamin A either structurally and/or functionally. It can be only obtained through diet and can exist in several forms such as retinol (preformed vitamin A), retinyl ester, or provitamin A carotenoids (β-carotene) having pleiotropic functions in development and disease. Retinoids are implicated in embryogenesis, reproduction, inflammation, proliferation, differentiation, and apoptosis by regulating large numbers of genes at the transcriptional level (Boylan et al., 1995). Retinoids are made up of three units: a bulky hydrophobic region, a linker unit and a polar carboxylic acid terminus (Figure 4). Retinol, one of the derivatives of vitamin A, is further metabolized to retinal by retinol dehydrogenase. Afterward, retinal is irreversibly oxidized to all-*trans* retinoic acid (ATRA) by retinal dehydrogenase (Freemantle, Spinella, & Dmitrovsky, 2003).

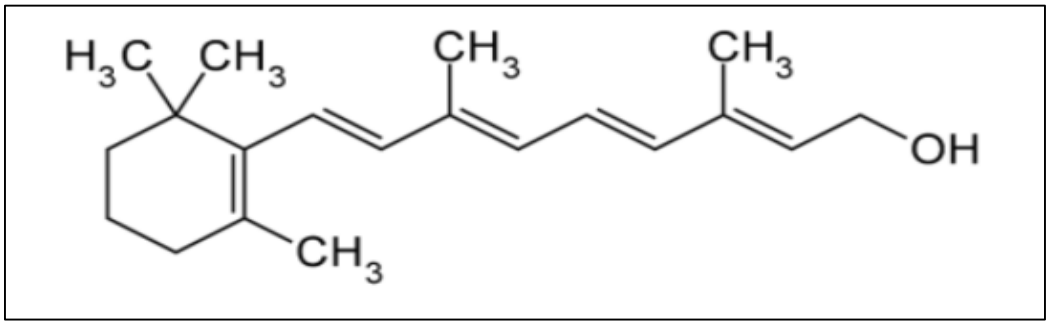

Figure 4. The basic chemical structure of the retinoid family.

2. All-trans Retinoic Acid

ATRA, also called tretinoin, has been extensively studied for the past several years and has made its way into clinic for the treatment of several types of cancers namely acute leukemia such as lymphoma, melanoma, lung cancer, cervical cancer, kidney cancer, neuroblastoma, glioblastoma and leukemias especially a rare one called acute promyelocytic leukemia (Sun, Yue, & Lotan, 2000). In addition, ATRA decreased breast cancer, gastric cancer, and colon cancer invasion *in vitro* and rhabdomyosarcoma metastasis in rats. ATRA, mediates its effect through the activation of the RARs exclusively which are known to dimerize with the retinoid X receptors (RXRs). Some evidence has shown that the retinoids inhibit metastasis in a variety of model systems. ATRA, was also proven to reverse the premalignant lesions in colorectal adenocarcinoma and other malignant disorders (Sun et al., 2000).

ATRA resistance and its side effects are common drawbacks seen particularly in patients treated with ATRA as a single drug. The incidence of acquired resistance declined after the combination of ATRA with other chemotherapeutic drugs. However, the chance of acquired resistance in patients who relapse from combination ATRA chemotherapy regimens despite a

restricted ATRA exposure is still present (Tomita, Kiyoi, & Naoe, 2013). In a recent study, ATRA resistance was seen *in vitro* on colorectal cancer cell lines (Gallagher, 2002).

3. 9-cis Retinoic Acid

ATRA is converted into 9-*cis* retinoic acid (9c-RA), also called alitretinoin, which is another endogenous retinoid having a biological activity (Christov et al., 2002) (K. Wu et al., 2000). 9c-RA is different from ATRA in that it activates both RARs and RXRs. It has been shown to be effective in the prevention of mammary and prostate cancer and as a topical treatment for Kaposi's sarcoma (Baumann et al., 2005). In addition, 9c-RA plays a role in regulating not only nuclear genes, but also mitochondrial gene transcription (Y. W. Lin et al., 2008).

ATRA and 9c-RA can induce apoptosis of human liver cancer cells (Wan, Cai, Cowan, & Magee, 2000), however little is known on the role of ATRA in colorectal cancer cell lines taking into account the ATRA resistance phenomenon, knowing that 9c-RA showed pro-apoptotic and anti-proliferative effects (Ocker, Herold, Ganslmayer, Hahn, & Schuppan, 2003).

F. Retinoid Binding Proteins

The general role of these retinoid binding proteins is to solubilize and stabilize retinoids in aqueous cellular spaces. Each retinoid binding protein has a distinct function in regulating transport and metabolism of certain retinoids (Bushue & Wan, 2010). Various isomeric forms of retinoids are associated with multiple types of retinoid binding proteins which are located either in the intracellular or in the extracellular compartment of the cell. Therefore, retinoids could be associated with cellular membranes or bound to a certain specific retinoid binding protein.

Retinoid binding proteins along with nuclear receptors are key mediators for the action of retinoids (Wolf, 2007).

1. Retinol Binding Protein

The main storage organ of vitamin A and main site of synthesis of retinol binding protein is the liver. The secretion of the retinol binding protein is regulated by the availability of retinol (Yang et al., 2005). Retinol circulates in blood while bound to the retinol binding protein which in turn binds to a small protein namely transthyretin. This complex transports retinol in circulation and delivers it to its target tissues (Seeliger et al., 1999).

2. The stimulated by retinoic acid gene 6

The stimulated by retinoic acid gene 6, STRA6, is a transmembrane protein which encodes for the cell surface RBP receptor whereby retinol binding protein binds and mediates the uptake of retinol from holo- retinol binding protein (Kawaguchi et al., 2007). It has been demonstrated that STRA6 is upregulated in mouse mammary gland tumors as well as in human colorectal tumors (Szeto et al., 2001). It is also implicated in many other diseases such as anophtalmia, congenital heart defects, mental retardation among others. Thus, these finding suggest that this transmembrane protein may be involved in diverse fields in addition to retinol binding protein transport.

3. Cellular retinol binding protein

Cellular retinol binding protein, CRBP, belongs to the family of fatty acid binding proteins in which it is tissue specific. For example, CRBP-II is expressed exclusively in the enterocytes of the intestine whereas CRBP-I and III are expressed constitutively in embryonic and adult tissues

(E et al., 2002). CRBP-I has a high affinity for retinol and contributes to the homeostasis of retinoids. This binding protein is commonly epigenetically silenced in the majority of cancers (Esteller et al., 2002). This leads to a reduction in the availability of retinyl esters in blood and slows down the body's ability to metabolize retinol (Matt et al., 2005).

4. Cellular retinoic acid-binding protein

The cellular retinoic acid-binding protein isoforms I and II (CRABP I and II) are known to display a high affinity for ATRA. In humans, they are conserved among species and have an average of 74% similarity in their sequences. The biological functions of the CRABP I and II are still poorly understood. In mouse knockout models, their disruption led to defects in limb development (Fawcett et al., 1995). Thus, they can have a role in the generation of adequate retinoic acid (RA) concentration gradients necessary for the development of limb bud. Most importantly, an increased expression of the CRABP I contributes to retinoic acid resistance of cancer cells. For that reason, in depth studies should be performed in order to examine carefully the exact implication of CRABPs on cancer therapy (Blaese, Santo-Hoeltje, & Rodemann, 2003). Based on the above, when retinoids are used as a mean of treatment for several diseases, the effect on binding proteins must be considered and well established for better understanding of the action of retinoids relative to each disease state.

G. Retinoic Acid Receptors

ATRA binds to the steroid hormone nuclear receptor superfamily: the RARs and the RXRs, having each three isoforms, α, β and γ. RARs form heterodimers with the RXRs affecting gene expression by binding to the retinoic acid response elements in target genes (Figure 5). This is called the retinoic acid classical pathway where its activation triggers cellular

differentiation, cell cycle arrest, and subsequently apoptosis (Gallagher, 2002). RAR-RXR heterodimers actively repress transcription in the absence of ligand. Upon binding to a ligand, the receptors dissociate from the corepressors and bind to receptor coactivators, and thus facilitate the recruitment of ribonucleic acid (RNA) polymerase-II and the basal transcription machinery. RXRs must be present in order to coordinate various actions of nuclear receptors, therefore, making it a master regulator in determining the effects of other hormones such as vitamin D_3 hormone (K. Wang, Chen, Xie, & Wan, 2008) (Chen, Wang, & Wan, 2010). Moreover, RXR can form heterodimers with other receptors such as estrogen receptors α, AP-1 receptor, peroxisome proliferator-activated receptors, and many others. By doing so, they regulate their partner receptors' pathway, and this is called the non-classical pathway (K. Wang et al., 2006).

Retinoic acid regulates gene expression by binding to its nuclear receptors which in turn activates the transcription of the downstream target genes. Therefore, retinoids exert their functions primarily by regulating gene expression. Until recently, non-genomic effects of RA have been uncovered (Schenk, Stengel, & Zelent, 2014).

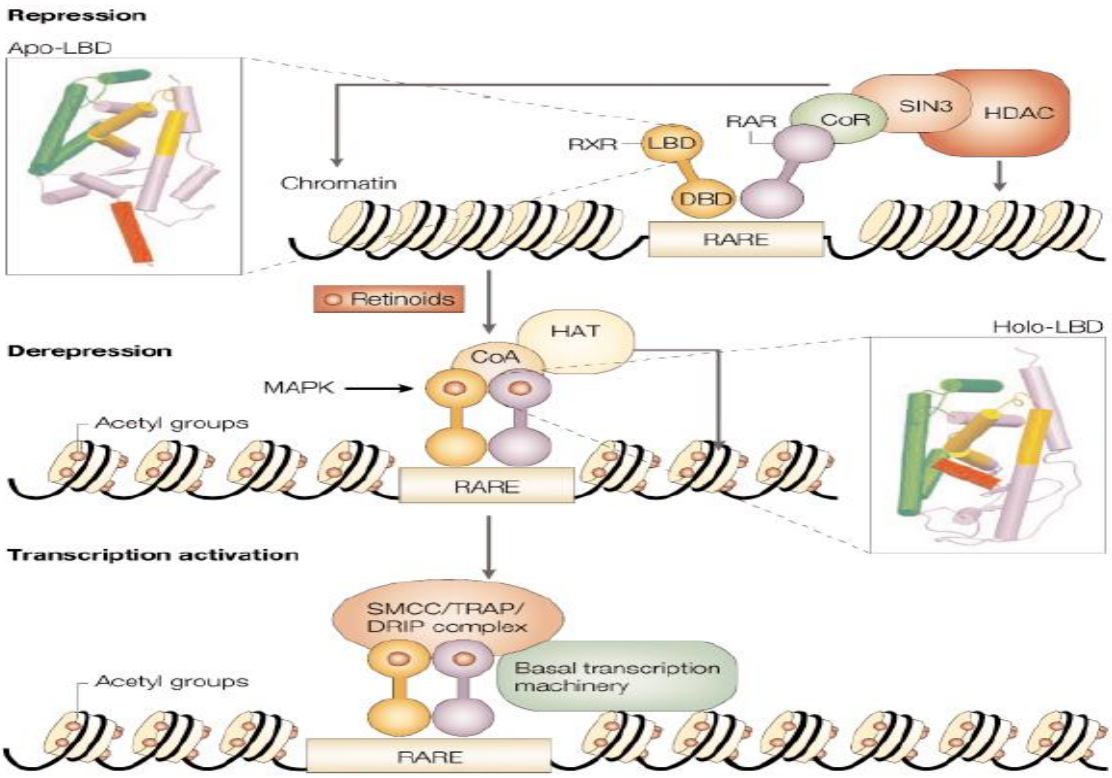

Figure 5. Mechanisms of transcriptional repression and activation by RAR–RXR. Adopted from (Altucci 2001).

H. Retinoids and Cancer

The role of retinoids in the field of cancer, as chemotherapeutic agents or chemopreventive agents, constitutes an ongoing area of research because of their anti-proliferative, differentiation, pro-apoptotic, and anti-oxidant effects. Epidemiological studies proved that a low level of vitamin A may lead to the development of cancer. The clinical usage of natural retinoids in the treatment of several tumors and hematological malignancies is limited and this due to several setbacks such as undesirable side effects and resistance to treatment. Retinoid administration is associated with teratogenicity, headaches, bone toxicity and serum triglycerides elevation (Germain et al., 2006).

Moreover, retinoids have been shown to suppress carcinogenesis in tumorigenic animal models for skin, oral, lung, breast, bladder, ovarian, and prostate (Bukhari et al., 2007) (Y. Wang et al., 2009) (Pisano et al., 2007) (Shah, Valdez, Wang, & Shapshay, 2001) (Y. Li et al., 2008) (Moon et al., 1994) (Liu et al., 2005). However, not a lot of studies have been done so far on colorectal cancer. In fact, the loss of RARβ, specifically $RAR\beta_2$ expression, is known to be associated with tumorigenesis and retinoid resistance, therefore making RARβ a predictive marker for a better prognosis in colorectal cancer patients. On another hand, $RAR\beta_4$ expression is increased in many types of cancer, one of which is colorectal cancer, and ultimately favors the progression and growth of tumor cells. Furthermore, weak RARγ expression was found to be an indicator of a poor clinical outcome in colorectal cancer patients (Perraud et al., 2011). In humans, retinoids reverse premalignant epithelial lesions, play a role in preventing lung, liver and breast cancer and finally induce the differentiation of myeloid cells (Muto, Moriwaki, & Shiratori, 1998) (Recchia et al., 2009) (Edelman et al., 2005) (Yamane et al., 2009). The mechanisms behind the changes in RAR expression are not well defined, which might be of great importance in order to improve patient care and treatment of colorectal cancer, the third most common cause of death in the world (Colombo et al., 2006).

I. Aim of the Study

The link between chronic inflammation, cell proliferation, and cancer in particular colorectal cancer is well established (Figure 5). However, few studies have addressed the role of bradykinin and retinoid signaling in human colorectal cancer cells. There is evidence linking mitogenic kinin peptides to the progression of tumors (Bhoola et al., 2001).

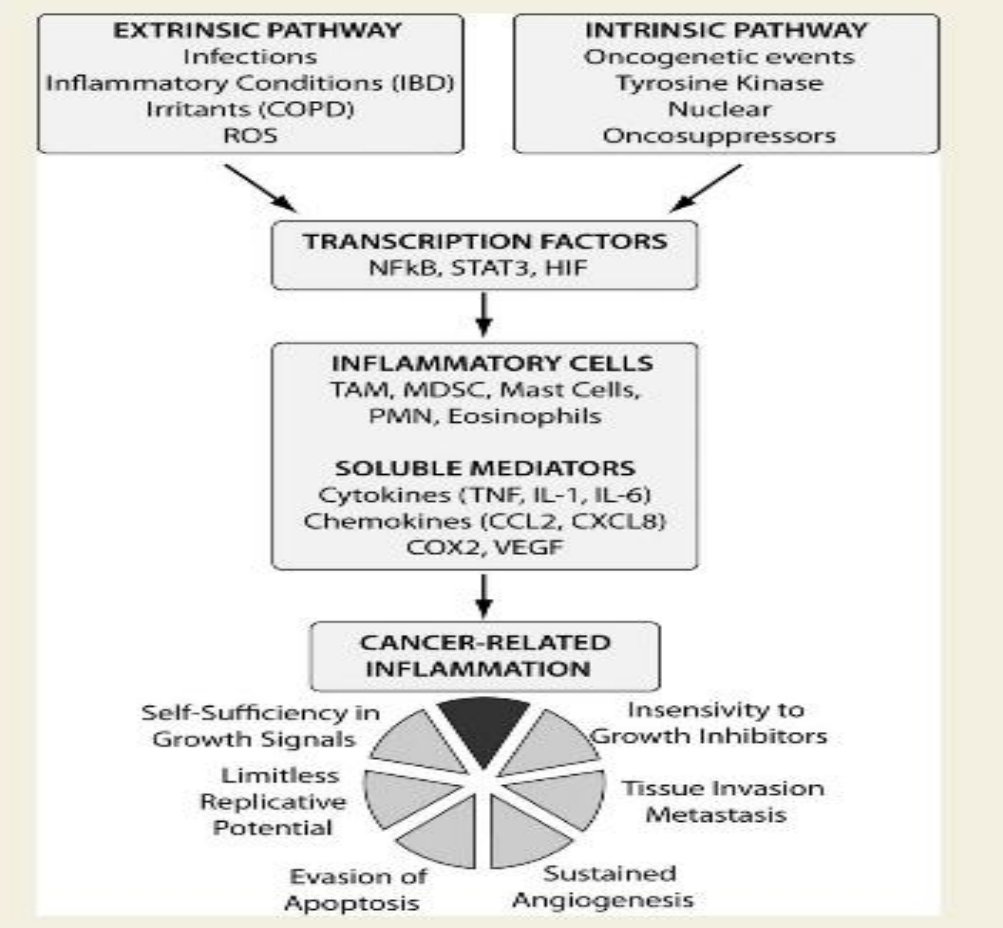

Figure 6. Molecular pathways linking inflammation and cancer. Adapted from (Del Prete et al., 2011).

Accordingly, we aim to use an *in vitro* human colorectal cancer model with cell lines having different *p53* and *p21* status to explore the possible crosstalk between bradykinin and any retinoic acid receptors signaling. This research may ultimately be a potential target for chemotherapeutics in the treatment of colorectal cancer. Towards the end we aim to:

- Assess the effect, after treatment with B1R agonist [Lys-des-Arg (9)-BK], B2R agonist (bradykinin), on the growth of colorectal cancer cell lines.
- Profile the basal transcript and protein levels of B1R, B2R and the retinoid receptors RARα, RARγ and RXRα in the different human colorectal cancer cell lines.
- Investigate the effects of B1R and B2R agonists' treatment on the expression of B1R, B2R, RARα, RARγ, and RXRα.
- Explore downstream signaling cascades of the bradykinin system such as ERK1/2 and protein kinase B (AKT).
- Determine the subcellular localization of the B2R and RARγ in the human colorectal cancer cell lines following different starvation conditions and bradykinin signaling.

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

1. In Vitro Human Model of Colorectal Cancer

Three human colorectal cancer cell lines: HCT116, HCT116 *p53*$^{-/-}$, HCT116 *p21*$^{-/-}$ were used.

HCT116

Malignant human epithelial colorectal cells isolated from patients with colonic carcinoma, and having a mutation in codon 13 of the *Ras* proto-oncogene with an abnormal expression in the transforming growth factor β 1 and 2 (TGFβ 1 and 2) which regulates proliferation, differentiation, adhesion, migration therefore contributing to the development of colorectal cancer (Ferrandiz et al., 2009).

HCT116 *p53*$^{-/-}$

This cell line responds to upstream signals by activating transcriptional genes crucial for cell cycle arrest, DNA repair, and apoptosis. The *p53*$^{-/-}$ gene, is the most common human gene which is deregulated in human cancers. Consequently, when the guardian of the genome, is knocked out, a high resistance to apoptosis occurs. Therefore, HCT116 p53$^{-/-}$ cell line is considered relatively more resistant to drugs than HCT116 or HCT116 p21$^{-/-}$ (Ferrandiz et al., 2009).

HCT116 *p21*$^{-/-}$

Cyclin-dependent kinase (CDK) inhibitor, p21, functions as a regulator of cell cycle progression at G1 and S phases. The *p21*$^{-/-}$ gene, is considered one of the most common deregulated tumor suppressor gene in human cancer. This cell line has an overexpression of the

wild type *p53* gene due to the enhanced stability of the p53 protein specifically in this subtype of cells. Therefore, HCT116 $p21^{-/-}$ cancer cells are less resistant and more sensitive to drugs than HCT116 $p53^{-/-}$.

Finally, all HCT116 cell lines have the same phenotype and, therefore, cannot be linked to a certain cancer stage. All are considered having an advanced malignancy (Ferrandiz et al., 2009).

2. Cell Culture Conditions

The HCT116 cells were cultured in RPMI media with its additives: 10% fetal bovine serum (FBS), 5% penicillin/streptomycin, and 5% sodium pyruvate. Whereas HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells were cultured in DMEM media with its additives: 10% FBS, 5% penicillin/streptomycin, 5% sodium pyruvate, and 5% non-essential amino acids. Then all cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide (CO_2). Cells can be used up to passage 80 in all experiments. Quiescence was achieved by transferring semi-confluent (60–80%) cells to serum-free DMEM or RPMI media for 24 hours before agonist stimulation: Bradykinin [10^{-7} M], B1R agonist [10^{-7} M], at different time points (6 hours, 24 hours, and 48 hours). For use in experimentation, an aliquot of stock B1R agonist and Bradykinin [$1x10^{-7}$ M] were serially diluted in 1ml acetic acid.

B. MTT Proliferation Assay

HCT116, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$ were seeded in triplicates into 96-well plates at a density of 5×10^3 cells/well. Cells were treated with different concentrations [10^{-6} M to 10^{-9} M] of bradykinin and B1R agonist, and ST1926 [10^{-6} M] as positive control for 24, 48, and

72 hours. Proliferation was assessed using thiazolyl blue tetrazolium bromide (MTT) dye *versus* control (sigma). In this step, metabolically active/viable cells had the ability to convert the yellow tetrazolium salt (MTT) into insoluble purple formazon crystals due to the high levels of nicotamide adenine dinucleotide (NADH) and nicotamide adenine dinucleotide phosphate (NADPH), which is a measure of mitochondrial metabolic activity. Finally, after overnight incubation, the reduced MTT optical density (OD) was measured at a wavelength of 595 nm using an ELISA reader (Multiskan Ex). The percentage cell viability was expressed as percentage growth relative to DMSO control wells and treated wells at indicated concentrations. The results represent the average ± standard error of three independent experiments. ST1926 was obtained from Biogen Institute (Ariano Irpino, Italy) and was reconstituted in 0.1% dimethylsulfoxide (DMSO) at a concentration of $1x10^{-2}$ M, aliquoted, stored at –80^{o}C, and used up to six months. For use in experimentation, an aliquot of stock ST1926 [$1x10^{-2}$ M] was serially diluted in 0.1% DMSO.

C. RNA Extraction and Real-Time PCR

Total RNA was extracted from the different cultured cell lines, using Ribozol (Amresco) according to the manufacturer's instructions. RNA concentration was measured at 260 nm, and the 260/280 ratio was determined using the nanodrop (ND) spectrophotometer ND-1000.

RT-qPCR: 1 µg of total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad), according to manufacturer's instructions. cDNA amplification reaction was done using the iQ SYBR green mix kit (Bio-Rad), according to the manufacturer's instructions. Human primer sequences for the genes of interest are shown in table 1 below.

Genes	Forward primer	Reverse primer
Bradykinin Receptor 1	ACATTCCTGCTGCGATCCAT	CCAGTGGTAGGAGGAAACCC
Bradykinin Receptor 2	CTGTTCGTGAGGACTCCGTG	GGGCAAAGGTCCCGTTAAGA
RARα	CACACCTGAGCAGCATCACA	CGGTCCTTTGGTCAAGCAGT
RARγ	GAGGAGCCCGAAAAAGTGGA	CCGGAGGTCGGTGATTTTCA
RXRα	AACATTTCCTGCCGCTCGAT	GGGTGCTGATGGGAGAATGC
β actin	CTCACCATGGATGATGATATCGC	AGGAATCCTTCTGACCCATGC

Table 2. Human primer sequences for bradykinin receptors, retinoic acid receptors and β actin.

PCR was performed using the iCycler iQ (BioRad) programmed for a 1 minute denaturation at 98 ^{o}C (1 cycle) followed by 40 cycles for: 9 seconds at 95 ^{o}C, 12 seconds annealing, 9 seconds extension at 72 ^{o}C, and finally one cycle of 10 minutes extension at 72 ^{o}C. After amplification, samples underwent melting to check the purity and integrity of the amplified samples. A standard curve for qPCR was prepared for B1R and B2R, RARα, RARγ, RXRα, and β actin.

Standard cDNA concentrations of 50 M, 5 M, 5×10^{-1} M, 5×10^{-2} M, 5×10^{-3} M, 5×10^{-4} M, and 5×10^{-5} M were prepared by serial dilution.

D. Western Blotting

20–30 µg of soluble proteins obtained using the total lysis buffer, were separated by SDS-PAGE (10%) under reducing conditions and transferred to nitrocellulose membranes using a Trans-Blot Turbo (Bio-Rad) for 30 minutes. The membranes were blocked for 1 hour in tris buffer saline tween (TBS-T) (25 mM Tris pH 7.4, 0.15 M NaCl, and 0.1% Tween-20) then immunoblotted with primary antibodies (B1R and B2R, RARα, RARγ, and RXRα) and anti-rabbit, anti-mouse and anti-goat secondary antibody IgG conjugated to horseradish peroxidase (Abcam), at different optimized dilutions in TBST-3% bovine serum albumin (BSA). β-actin was measured in the same membranes by stripping the membrane and immunoblotting again. ERK 1/2 and AKT phosphorylation were assessed after blocking, by using a specific primary antibody (1:1000 dilution) followed by incubation with goat anti-rabbit alkaline phosphatase-conjugated IgG (Cell Signaling). The immunoreactive bands were visualized using the enhanced chemiluminescence reagent (ECL kit, Roche) according to the manufacturer's instructions. Membranes were exposed to Kodak film (MR, Kodak, USA), and bands were measured by densitometry and quantified by the ImageJ program.

E. Immunofluorescence

100,000 HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells were plated in 6-well plates onto glass coverslips coated with 0.1 mg/mL poly-D-lysine and maintained in DMEM or RPMI media containing 10% FBS and starved for 24 hours or 30 minutes according to the optimized conditions. Subconfluent cells were then washed with phosphate buffered saline (PBS)

containing 0.1% sodium azide and subsequently fixed with 2% formaldehyde for 30 minutes. Cells were washed again with PBS, permeabilized 20 minutes with 0.1% Triton-X-100 in PBS and blocked with 0.1% Triton-X-100 in PBS containing 2% BSA for 30 minutes at room temperature. Cells were then incubated overnight with a primary anti-rabbit antibody at dilutions 1:250 and 1:500 in blocking solution for RARγ and B2R respectively. Subsequently, cells were washed twice with PBS, and incubated with secondary anti-rabbit antibody conjugated to Fluorescein Isothiocyanate (dilution 1:500) in blocking solution for 1 hour at room temperature. Cells were washed twice with PBS, and coverslips were mounted using Prolong Diamond Antifade Mountant ®. Fluorescein Isothiocyanate fluorescence was excited using the Argon 488 nano meter laser. Cells were visualized under the confocal microscopy.

F. Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM) of 3 independent experiments and analyzed by using non-parametric tests as a complete randomized design using SigmaStat 3.1 Software. Differences are considered significant if: * $P < 0.05$, ** $p<0.01$, and ***$p<0.001$.

CHAPTER III

RESULTS

A. Basal Level Expression of Bradykinin and Retinoid Receptors in Colorectal Cancer Cells

We first determined the basal transcript and protein levels of the different bradykinin receptors (B1R and B2R) and retinoid receptors (RARγ, RARα, RXRα) in the three colorectal cancer cell lines, namely HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$. To do so, the mRNA levels of each receptor in the three cell lines were measured by RT-qPCR relative to β-actin mRNA levels and the ratio was normalized relative to the HCT116 cell line. As shown in Figure 6, the basal transcript levels of the different bradykinin and retinoid receptors were detected in the different colorectal cancer cell lines, and were not modulated by the *p53* or *p21* status of the tested cells.

The basal protein levels of the receptors of interest mentioned above were also measured by western blot analysis in the three colorectal cancer cell lines. Similarly to the transcript levels, the basal protein levels of the B1R, B2R, RARγ, RARα and RXRα were detected in the different colorectal cancer cell lines, and were not modulated by the *p53* or *p21* status of the tested cells (Figure 7).

In summary, p53 and p21 status does not affect the basal expression level of the bradykinin and retinoid receptors in the tested human colorectal cancer cells.

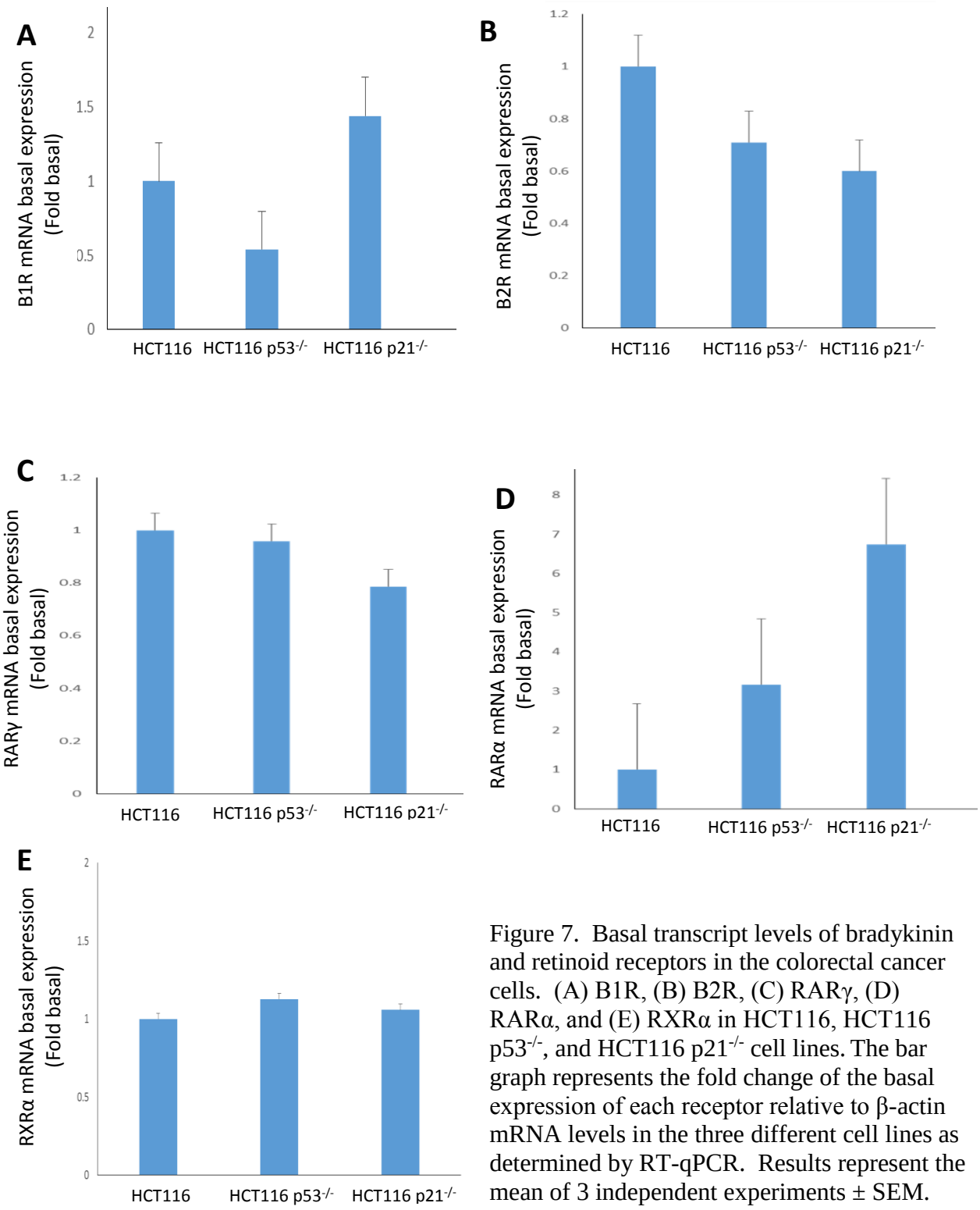

Figure 7. Basal transcript levels of bradykinin and retinoid receptors in the colorectal cancer cells. (A) B1R, (B) B2R, (C) RARγ, (D) RARα, and (E) RXRα in HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cell lines. The bar graph represents the fold change of the basal expression of each receptor relative to β-actin mRNA levels in the three different cell lines as determined by RT-qPCR. Results represent the mean of 3 independent experiments ± SEM.

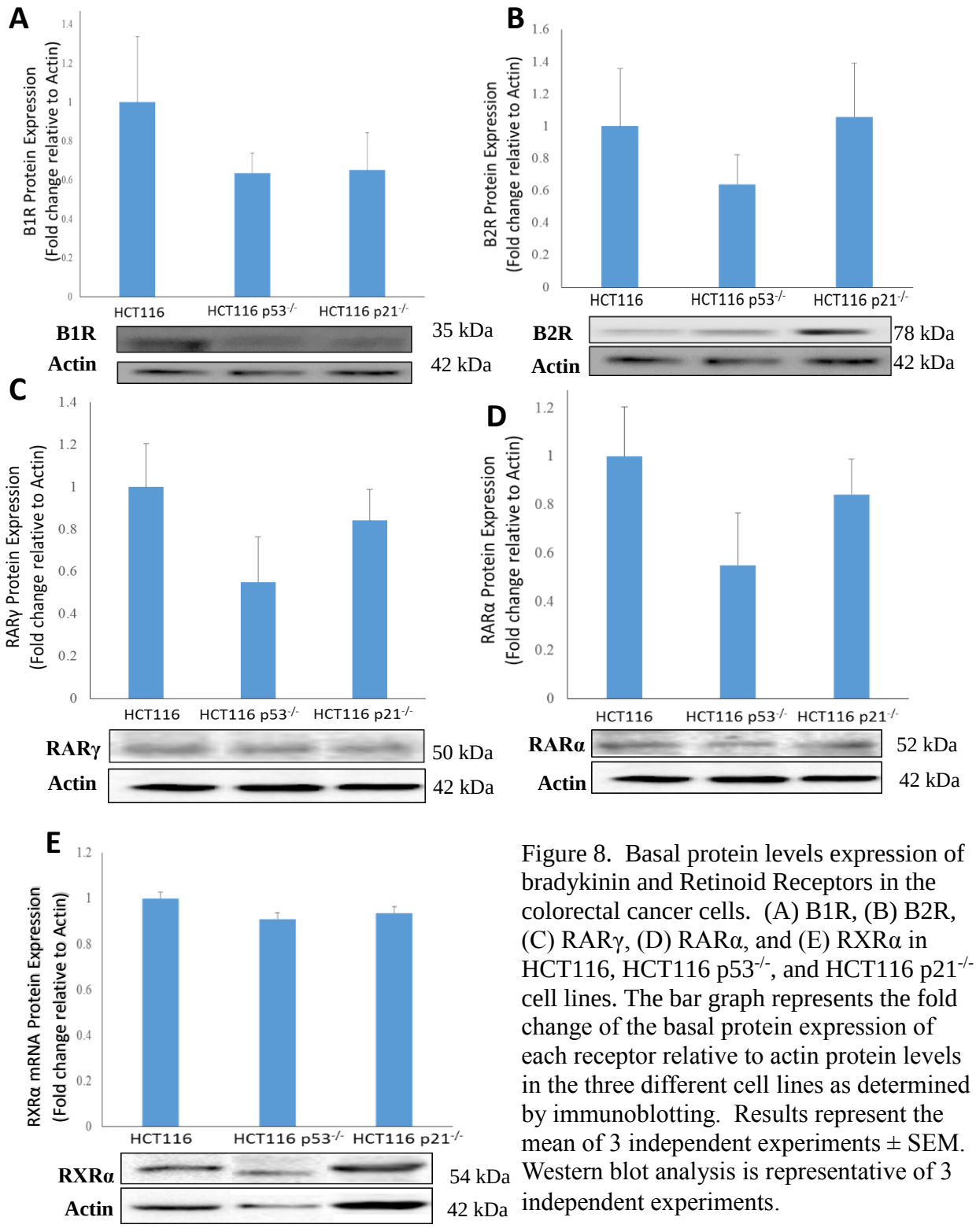

Figure 8. Basal protein levels expression of bradykinin and Retinoid Receptors in the colorectal cancer cells. (A) B1R, (B) B2R, (C) RARγ, (D) RARα, and (E) RXRα in HCT116, HCT116 p53$^{-/-}$, and HCT116 p21$^{-/-}$ cell lines. The bar graph represents the fold change of the basal protein expression of each receptor relative to actin protein levels in the three different cell lines as determined by immunoblotting. Results represent the mean of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments.

B. Regulation of Bradykinin 1 Receptor by Bradykinin 1 Receptor Agonist Treatment in Colorectal Cancer Cells

The effect of B1R agonist [Lys-des-Arg (9)-BK], on the mRNA levels of B1R is shown in Figure 8. To investigate whether B1R agonist has any effect on the gene expression level of B1R, we measured the level of B1R mRNA in the three different cell lines namely HCT116, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$cell lines after incubating them with the B1R agonist at 10^{-7} M for 6, 24, and 48 hours. In HCT116 cells, B1R agonist treatment induced significantly the gene expression of B1R at 24 hours of incubation (3 folds ± 0.7, B1R agonist *versus* control * $p<0.05$) then declined at 48 hours of incubation (0.5 folds ± 0.1, B1R agonist *versus* control * $p<0.05$) (Figure 8A). Furthermore, B1R agonist led to the induction of B1R transcripts post-treatment by 6 folds in the HCT116 $p53^{-/-}$ cell line then returned to basal levels by 24 hours (Figure 8B). Finally, in the HCT116 $p21^{-/-}$, B1R agonist led to a gradual increase in B1R mRNA expression at 6 hours to reach its peak at 24 hours however this difference was not statistically significant (Figure 8C).

The effect of B1R agonist on the protein levels of B1R is shown in Figure 9. Protein levels tested by immunoblotting on the colorectal cancer cells, were measured after treatment with B1R agonist for 6, 24, and 48 hours. In the HCT116 and HCT116 $p21^{-/-}$ cell lines, although treatment with B1R agonist at three different time points, increased the expression of B1R however, this increase was not significant (Figures 9A and 9C). In addition, B1R agonist treatment did not affect B1R protein levels in HCT116 $p53^{-/-}$ cells (Figure 9B).

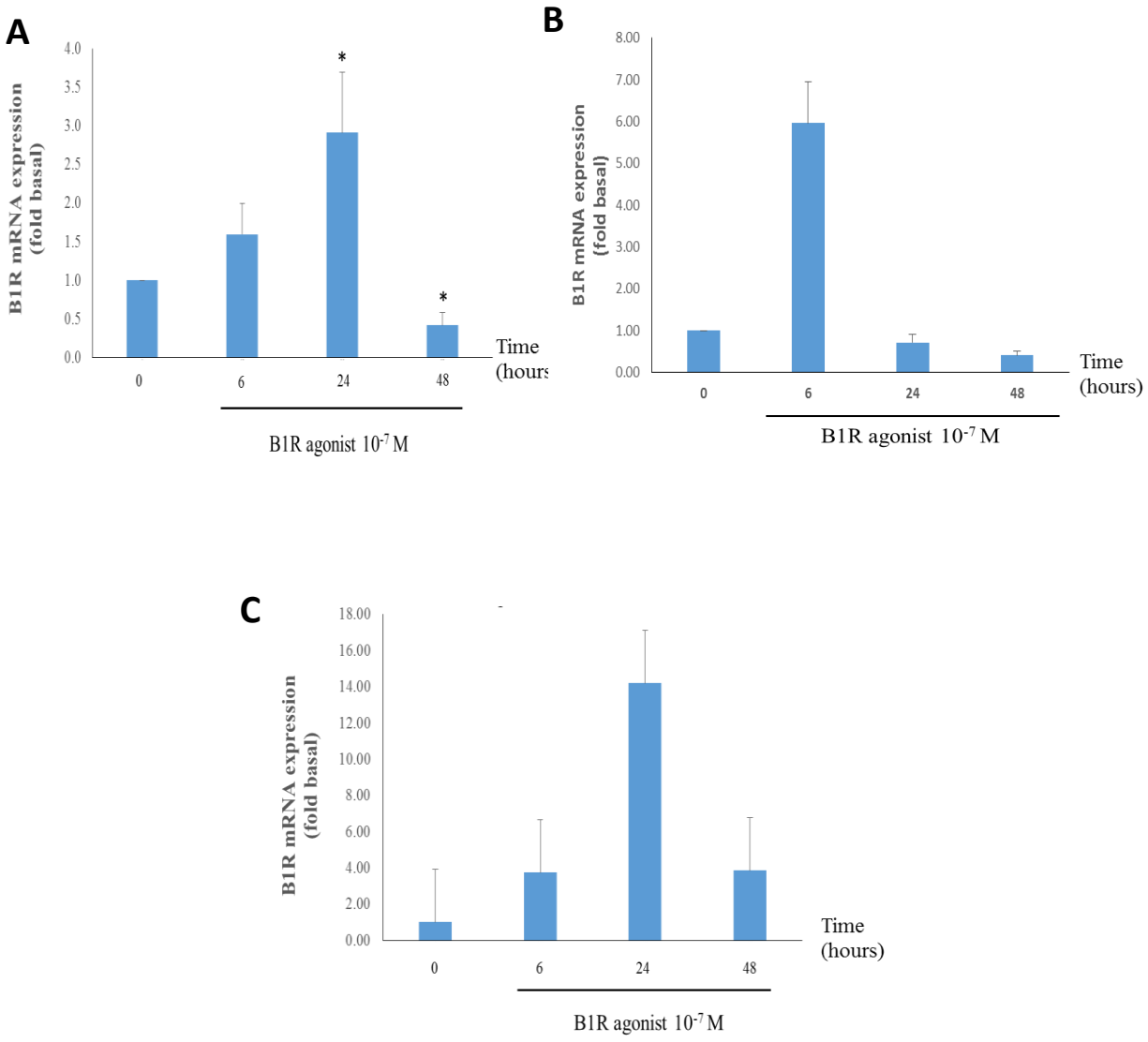

Figure 9. Regulation of B1R transcripts by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby B1R and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents the fold change of B1R relative to β-actin mRNA levels of 3 independent experiments ± SEM. Non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

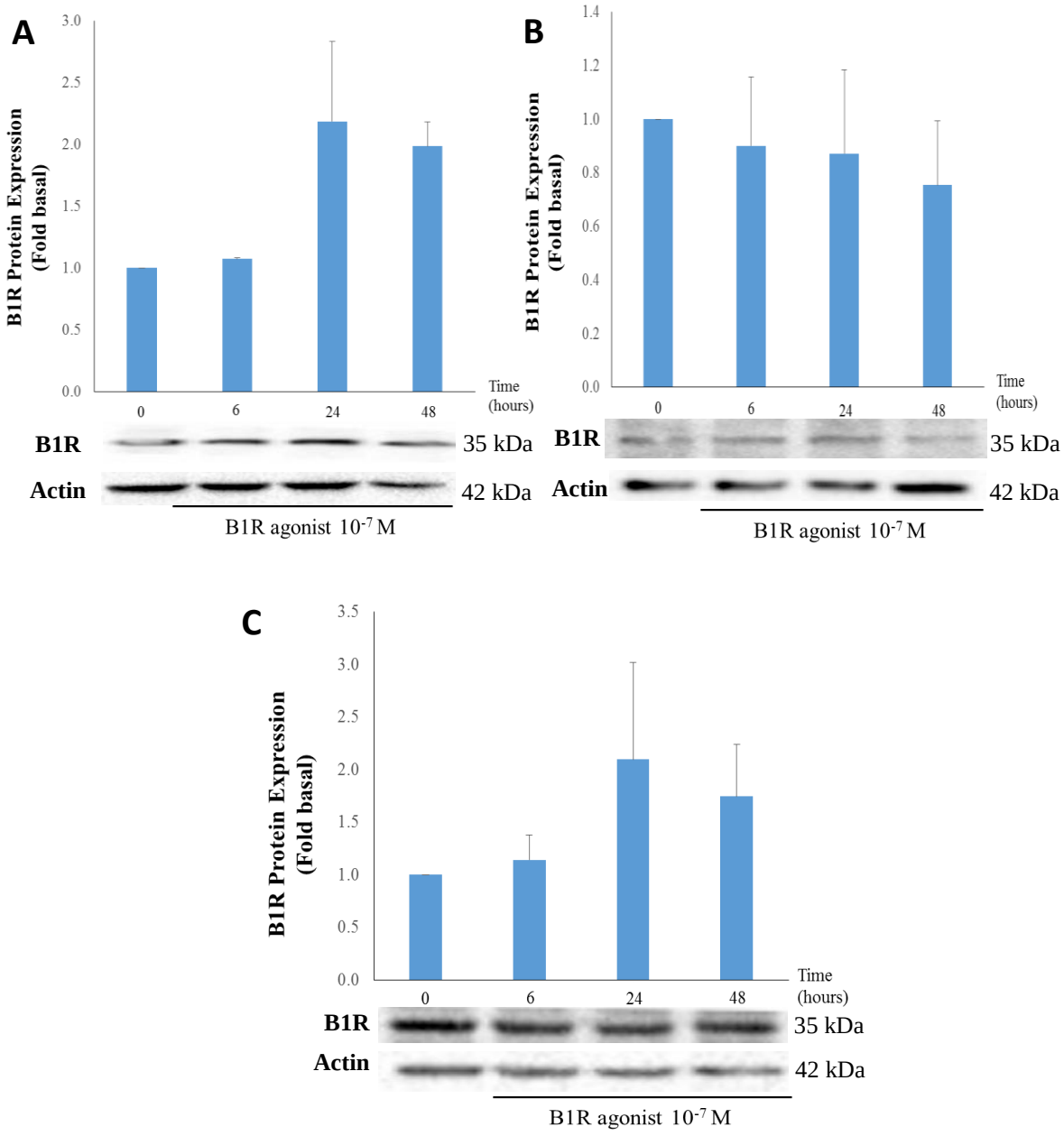

Figure 10. Regulation of B1R protein levels by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against B1R and actin antibodies, B1R and actin levels were measured by western blot analysis. The bar graph represents the fold change of B1R relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments.

C. Regulation of Bradykinin 2 Receptor by Bradykinin 1 Receptor Agonist Treatment in Colorectal Cancer Cells

To examine the effect of B1R agonist on the gene expression level of B2R, the levels of B2R mRNA were measured in the different cell lines after treatment with B1R agonist for 6, 24, and 48 hours. In the HCT116 cells, B1R agonist treatment for 6 hours led to a significant decrease in B2R mRNA expression (0.3 folds ± 0.1, B1R agonist *versus* control $*P<0.05$). These levels increased back at 24 and 48 hours post treatment (Figure 10A). However, no changes in B2R mRNA levels were observed in HCT116 $p53^{-/-}$ cells (Figure 10B). Interestingly, B2R mRNA levels were increased at 6 and 48 hours post treatment with the B1R agonist (5 folds ± 2 and 5.2 folds ± 0.9, respectively, B1R agonist *versus* control $*P<0.05$), however, decreased at 24 hours of treatment (0.7 folds ± 0.09, B1R agonist *versus* control, with no statistical significance) expressed relative to β-actin mRNA (Figure 10C).

Protein levels of B2R were also measured after incubation with B1R agonist for 6, 24, and 48 hours. The protein expression levels were significantly increased at 24, and 48 hours in HCT116 and HCT116 $p53^{-/-}$ but not in HCT116 $p21^{-/-}$ cells (Figure 11).

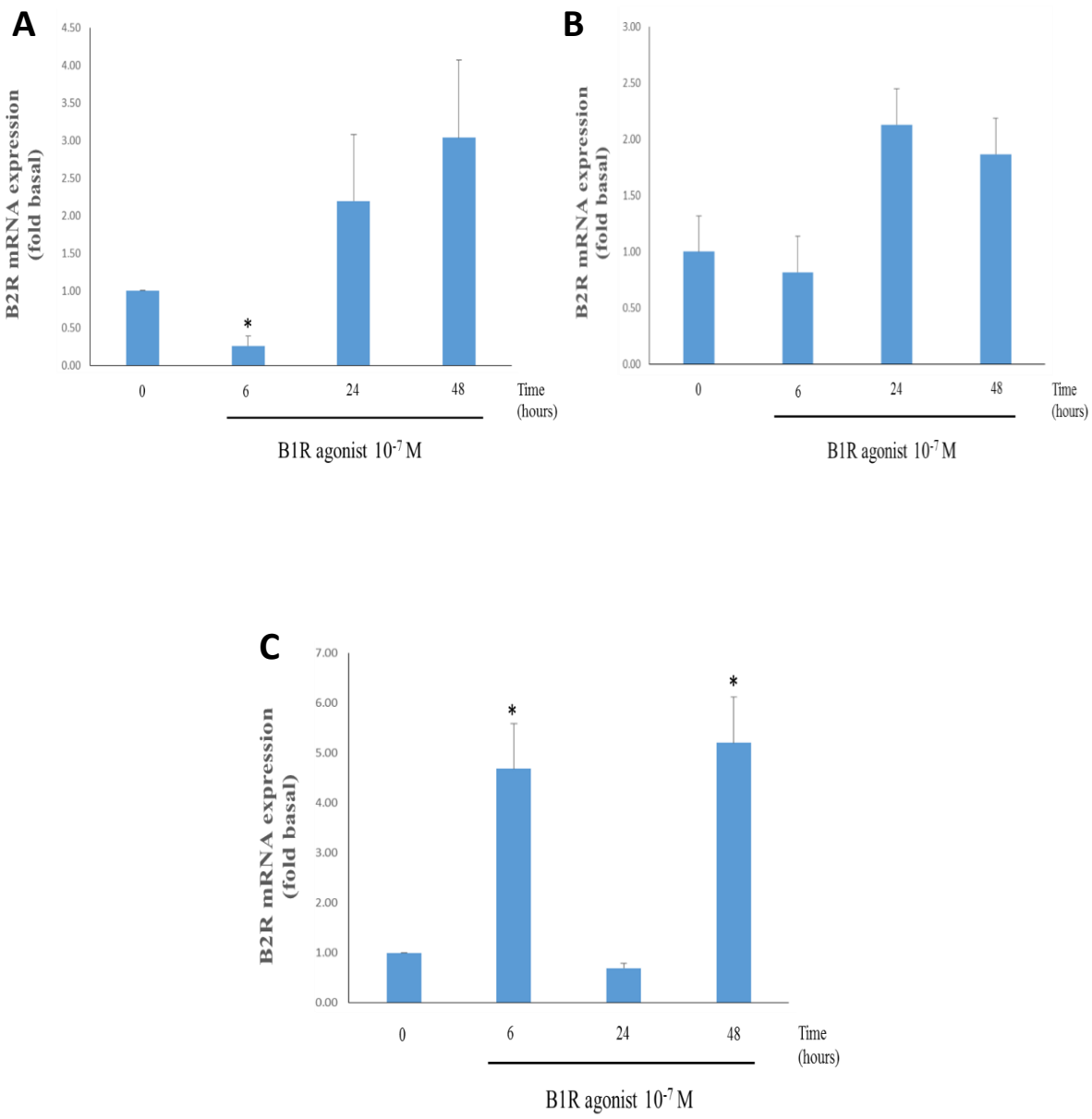

Figure 11. Regulation of B2R transcripts by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby B2R and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents the fold change of B2R relative to β-actin mRNA levels of 3 independent experiments ± SEM. Paired T test was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

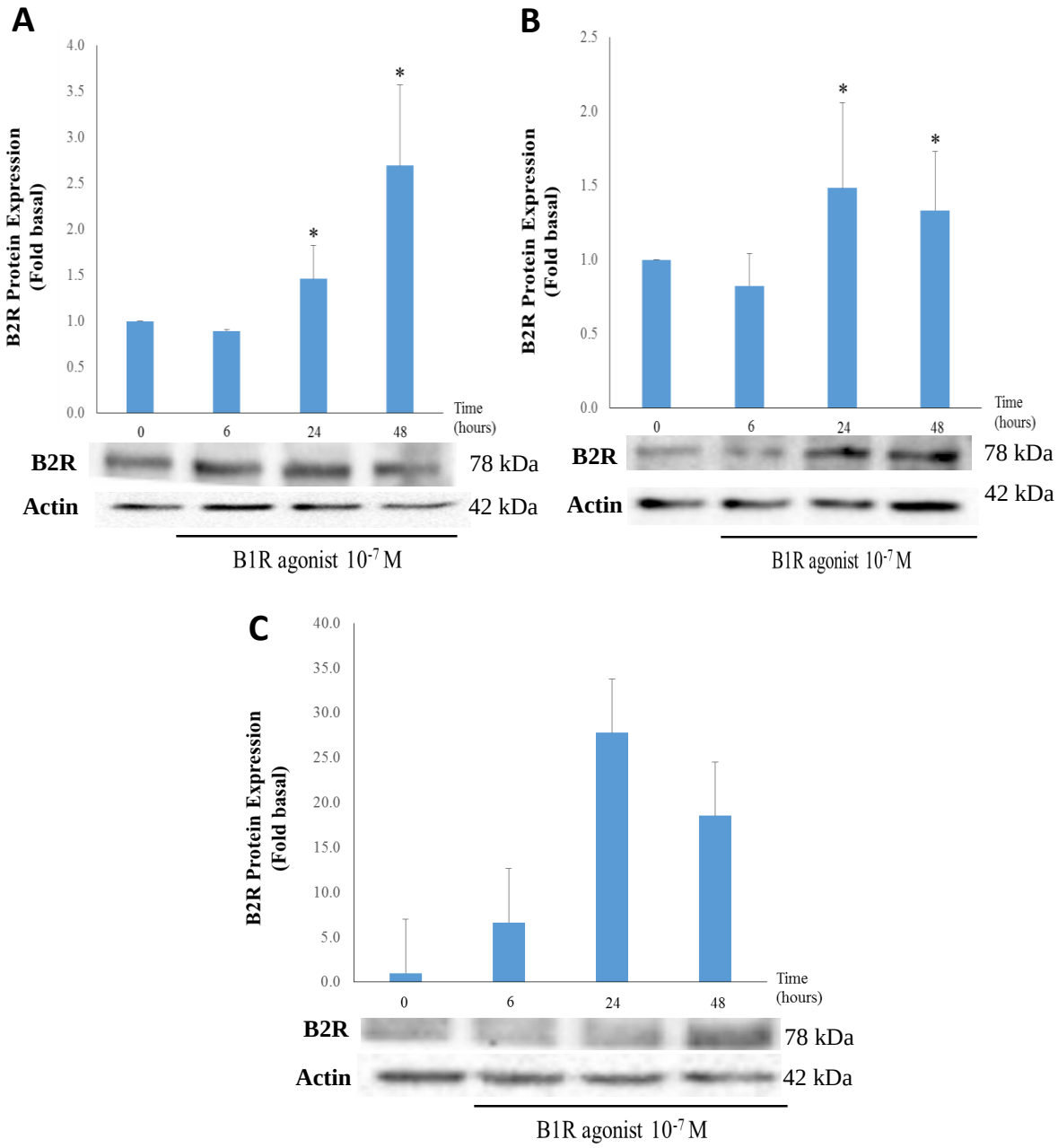

Figure 12. Regulation of B2R protein levels by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against B2R and actin antibodies, B2R and actin levels were measured by western blot analysis. The bar graph represents the fold change of B2R relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. One way ANOVA (A) and T test (B) were used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

D. Induction of Retinoic Acid Receptor gamma by Bradykinin 1 Receptor Agonist Treatment in Colorectal Cancer Cells

The effect of B1R agonist treatment on RARγ was evaluated by measuring the level of RAR gamma mRNA by RT-qPCR as presented in Figure 12. HCT116 cells incubated with B1R agonist have an increased expression of RARγ at 6, 24, and 48 hours (1.72 folds ± 0.5, 1 folds ± 0.5 and 1.24 folds ± 0.4 respectively, B1R agonist *versus* control) however, with no statistical significance (Figure 12A). As for HCT116 $p53^{-/-}$ cell line, the expression of RARγ upon treatment with B1R agonist was decreased at 6 hours (0.82 folds ± 0.3, B1R agonist *versus* control, with no statistical analysis) and was induced at 24 hours of incubation (2.17 folds ± 0.5, B1R agonist *versus* control, with no statistical significance) but maintained at 48 hours (1 fold ± 0.4, B1R agonist *versus* control) but with no statistical significance (Figure 12B). Finally, as for HCT116 $p21^{-/-}$ cell line, RARγ mRNA levels were significantly decreased at 6 hours of incubation (0.3 folds ± 0.2, B1R agonist *versus* control $*P<0.05$) and were slightly then increased at 48 hours (4 folds ± 0.5, B1R agonist *versus* control $*P<0.05$) expressed relative to β-actin mRNA (Figure 12C).

Moreover, the protein levels of RARγ were measured by immunoblotting after treatment with B1R agonist for 6, 24, and 48 hours and presented in Figure 13. B1R agonist treatment had no effect on RARγ protein levels up to 24 hours but significantly increased RARγ proteins at 48 hours in HCT116 cells (1.4 folds ± 0.4, B1R agonist *versus* control $*P<0.05$) (Figure 13A). In addition, the protein expression levels of RARγ were significantly increased at 6, 24, and 48 hours post treatment in HCT116 $p53^{-/-}$ cell line (2.1 folds ± 0.7, 3.7 folds ± 1.8 and 2.3 folds ± 0.9 respectively, B1R agonist *versus* control $*P<0.05$) (Figure

13B). Finally, B1R agonist increased the RARγ protein levels at 48 hours of treatment in HCT116 $p21^{-/-}$cell line (1.8 folds ± 0.2 respectively, B1R agonist *versus* control *P<0.05) (Figure 13C).

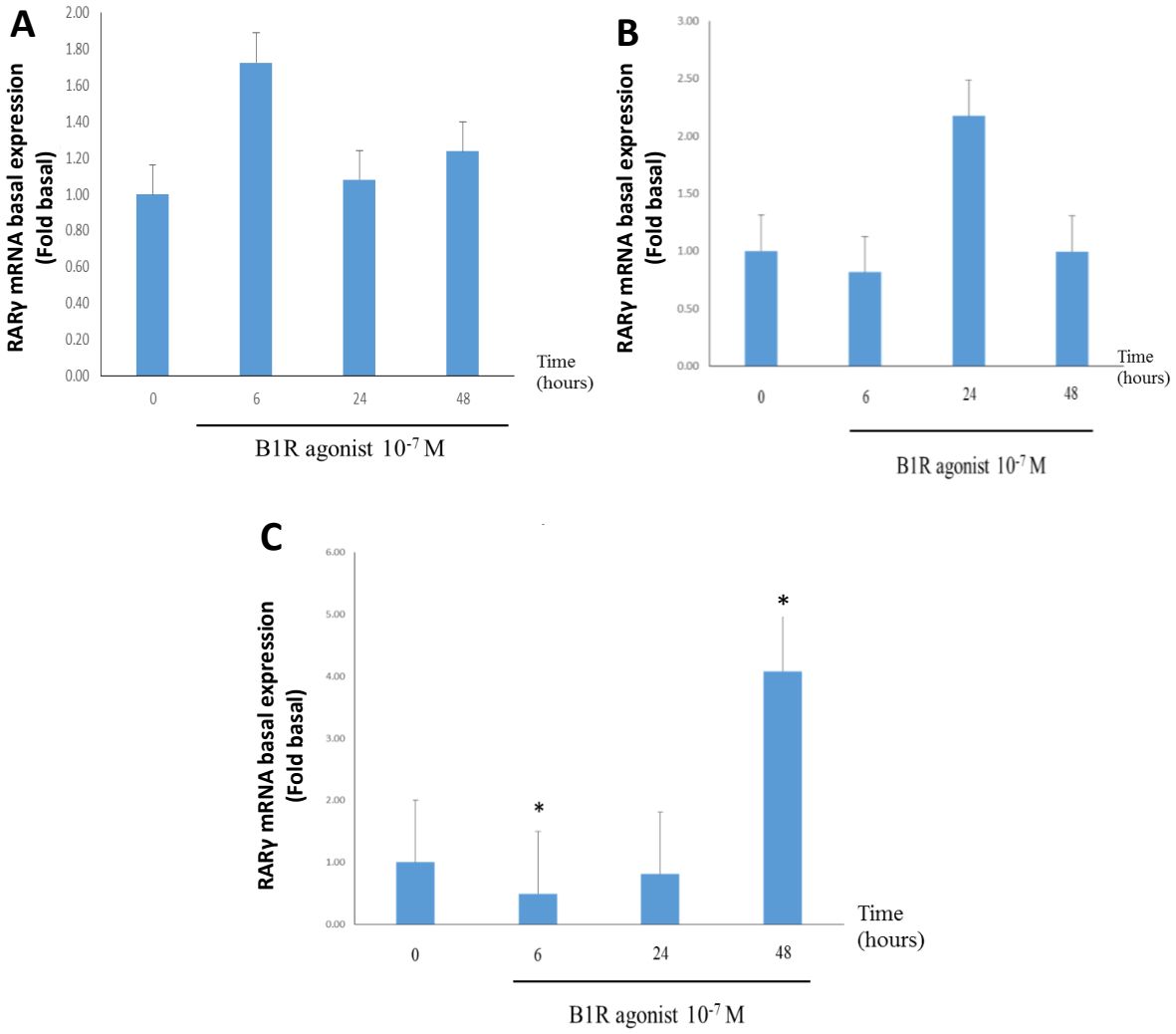

Figure 13. Regulation of RARγ transcripts by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby RARγ and β-actin levels were measured by RT-qPCR. The bar graph represents the fold change of RARγ relative to β-actin mRNA levels of 3 independent experiments ± SEM. T test was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

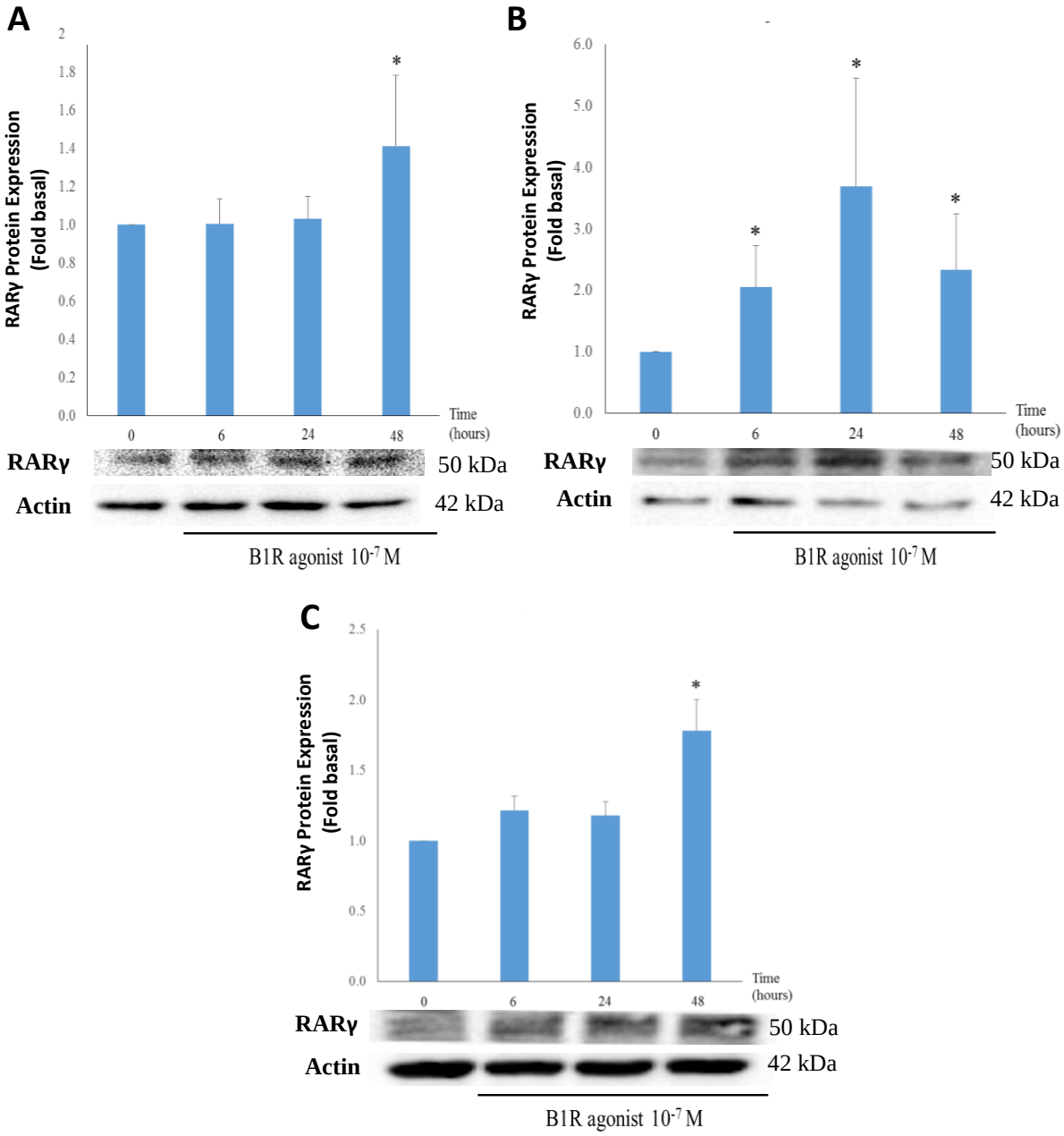

Figure 14. Induction of RARγ protein levels by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against RARγ and actin antibodies, RARγ and actin levels were measured by western blot analysis. The bar graph represents the fold change of RARγ relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. T-test (A), Paired T test (B) and Holm-Sidak method (C) were used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

E. Regulation of Retinoic Acid Receptor alpha by Bradykinin 1 Receptor Agonist Treatment in Colorectal Cancer Cells

The effect of B1R agonist on the mRNA levels of RARα is shown in Figure 14. B1R agonist treatment did not change significantly the levels of RARα transcripts in HCT116 cells (Figure 14A). However, RARα mRNA levels were significantly decreased at 6, and 24 hours (0.46 folds ± 0.1 and 0.48 folds ± 0.2 respectively, B1R agonist *versus* control *P<0.05) and significantly increased at 48 hours of incubation in HCT116 $p53^{-/-}$ cell line (2.1 folds ± 0.5, B1R agonist *versus* control *P<0.05) (Figure 14B). In the HCT116 $p21^{-/-}$cell line, the expression level of RARα mRNA was decreased at 6 hours (0.21 folds ± 0.2, B1R agonist *versus* control, with no statistical significance) and significantly decreased at 48 hours (0.4 folds ± 0.3, B1R agonist *versus* control *P<0.05) (Figure 14C).

Next, the protein levels of RARα were measured after treatment with B1R agonist for 6, 24, and 48 hours and shown in Figure 15. RARα protein levels were increased at 48 hours of treatment with B1R agonist in HCT116 cells (1.5 folds ± 0.3 folds respectively, B1R agonist *versus* control *P<0.05) (Figure 15A). As for HCT116 $p53^{-/-}$ cell line, RARα protein levels were increased at 24, and 48 hours post treatment (1.4 folds ± 0.3 and 2.5 folds ± 0.8, respectively, B1R agonist *versus* control *P<0.05) (Figure 15B). However, in HCT116 $p21^{-/-}$ cells, B1R agonist treatment at different time points did not change significantly RARα protein levels (Figure 15C).

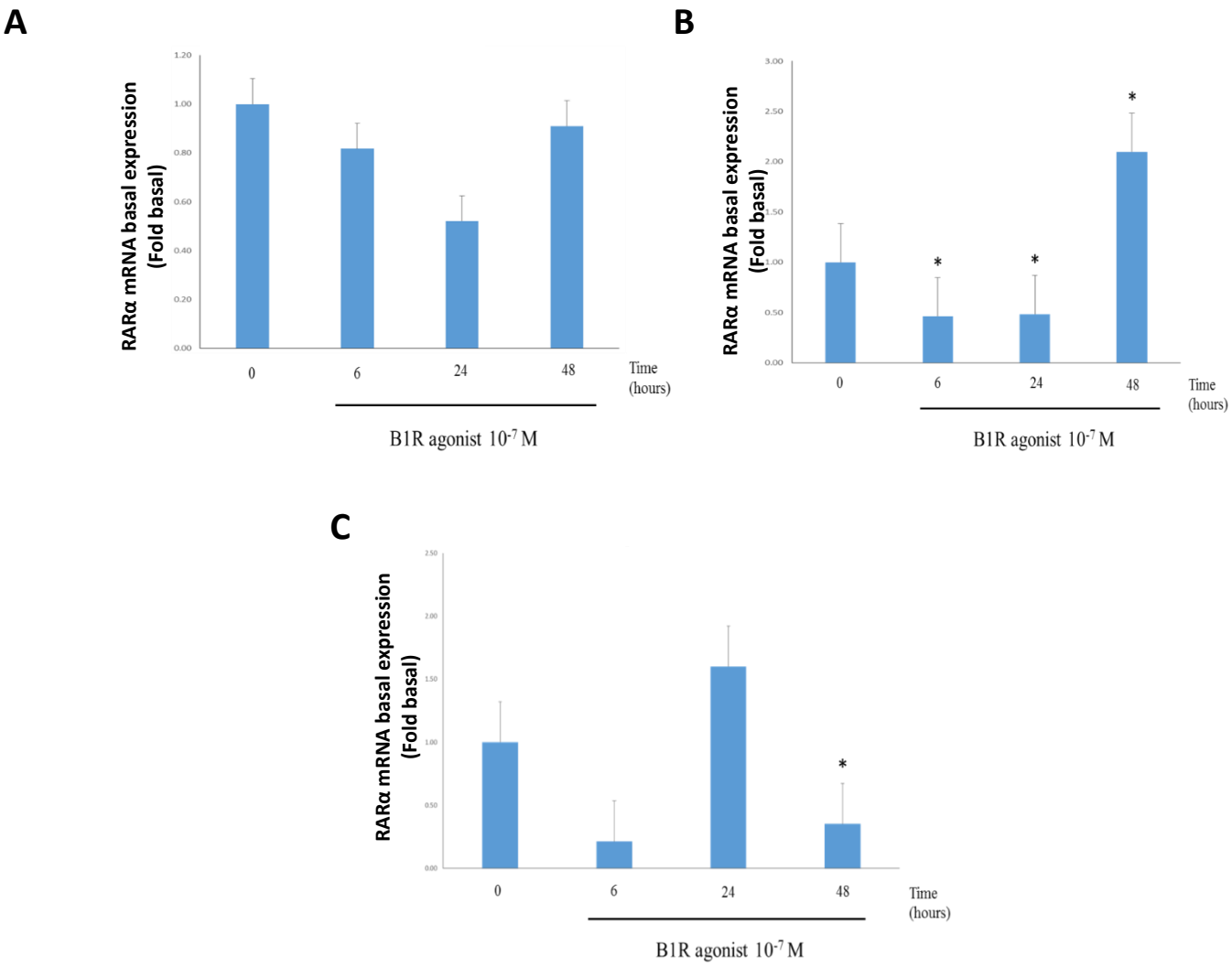

Figure 15. Regulation of RARα transcripts by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby RARα and β-actin levels were measured by RT-qPCR. The bar graph represents the fold change of RARα relative to β-actin mRNA levels of 3 independent experiments ± SEM. T test (B) and Mann-Whitney Rank Sum Test (C) were used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

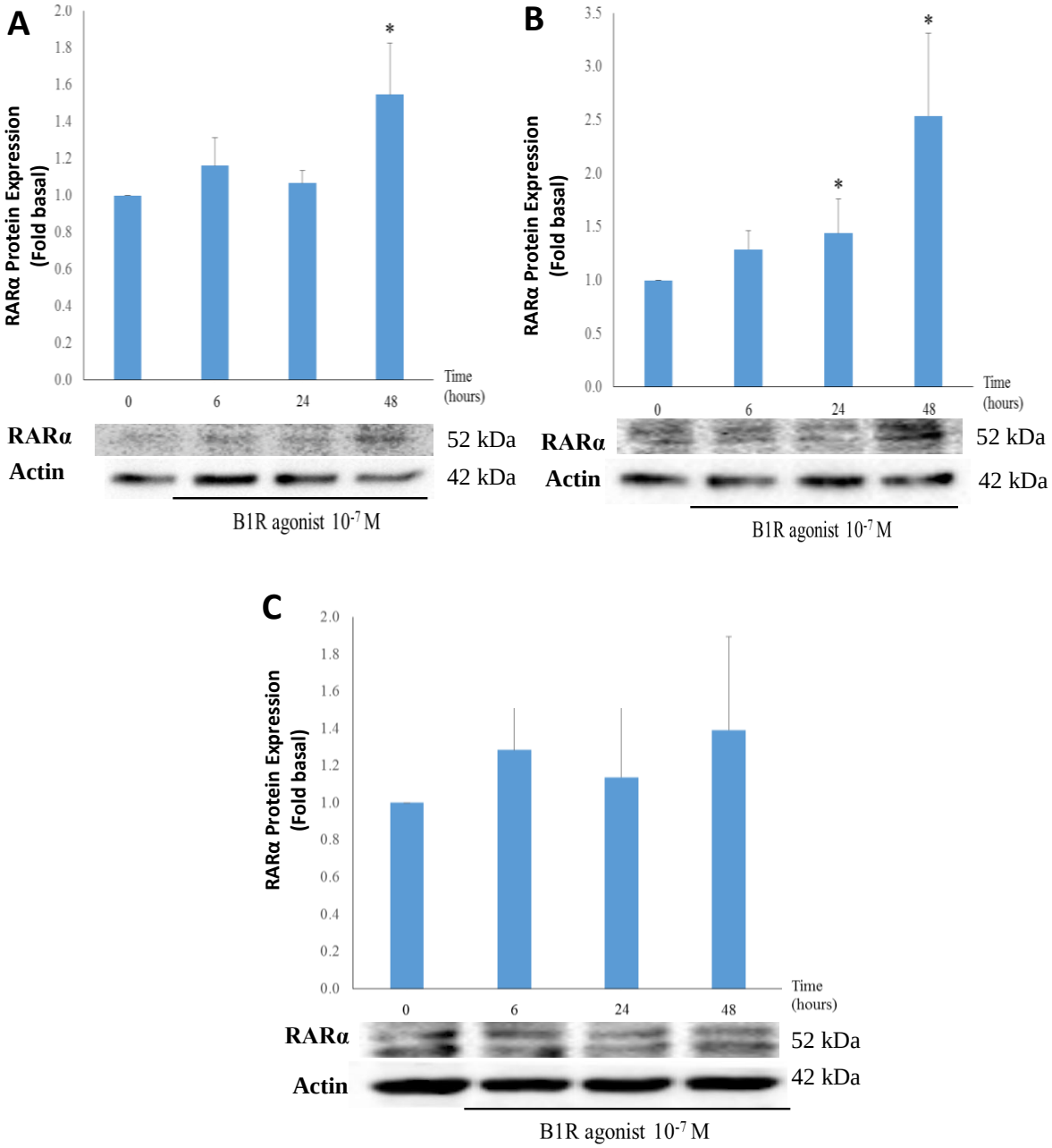

Figure 16. Regulation of RARα protein levels by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against RARα and actin antibodies, RARα and actin levels were measured by western blot analysis. The bar graph represents the fold change of RARα relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. T-test was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

F. Effect of Bradykinin 1 receptor Agonist Treatment on Retinoid X Receptor alpha Gene Expression in Colorectal Cancer Cells

The effect of B1R agonist treatment on the mRNA levels of RXRα is shown in Figure 16. The mRNA levels of RXRα in HCT116 cell line was found to be decreased at 6, and 24 hours of incubation with B1R agonist (0.22 folds ± 0.1 and 0.15 folds ± 0.1 respectively, B1R agonist *versus* control, with no statistical significance) and increased at 48 hours (4.72 folds ± 3.8, B1R agonist *versus* control, with no statistical significance) (Figure 16A). Moreover, B1R agonist was shown to increase the mRNA expression level of RXRα at 6, 24, and 48 hours of incubation in HCT116 $p53^{-/-}$ cell line (4.27 folds ±3, 2 folds ± 0.7 and 2.35 folds ± 0.8 respectively, B1R agonist *versus* control, with no statistical significance) (Figure 16B). Finally, the mRNA expression level of RXRα was increased at 6 and 48 hours of incubation (8.14 folds ± 7 and 6.65 folds ± 2 respectively, B1R agonist *versus* control, with no statistical significance) and was decreased at 24 hours in HCT116 $p21^{-/-}$ cell line (0.9 folds ± 1 B1R agonist *versus* control, with no statistical significance) (Figure 16C). In summary, B1R agonist treatment did not significantly alter RXRα transcript levels in the different colorectal cancer cells (Figure 16).

In addition, the protein levels of RXRα were measured after treatment with B1R agonist for 6, 24, and 48 hours and presented in Figure 17. B1R agonist treatment of HCT116 and HCT116 $p21^{-/-}$ did not significantly alter RXRα protein levels (Figures 17A and 17C). However, the same treatment significantly increased RXRα protein levels at 24 and 48 hoursin HCT116 $p53^{-/-}$ cells (1.9 folds ± 0.3 and 2.3 folds ± 0.4 respectively, B1R agonist *versus* control $*P<0.05$) (Figure 17B).

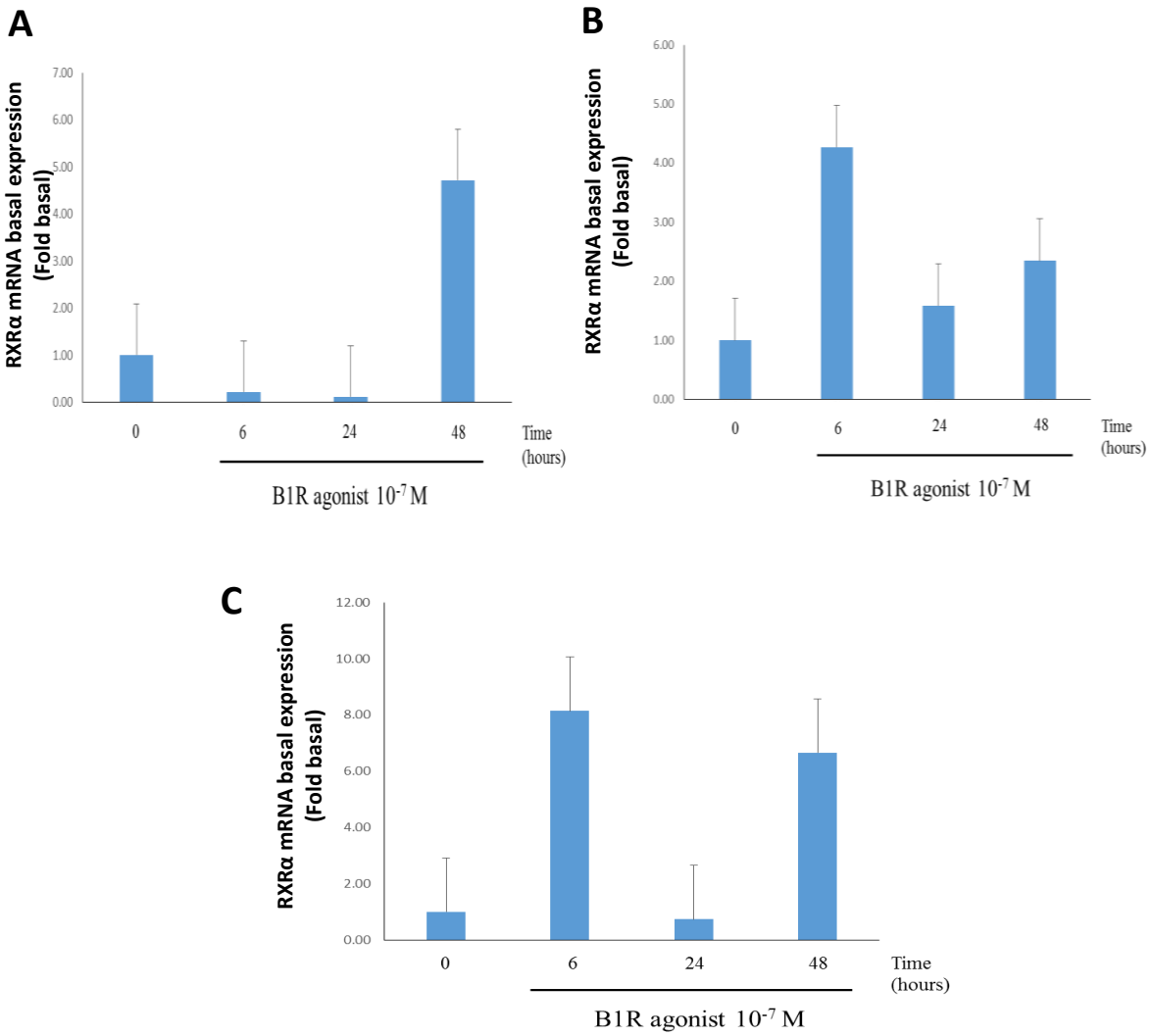

Figure 17. Regulation of RXRα transcripts by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby RXRα and β-actin levels were measured by RT-qPCR. The bar graph represents the fold change of RXRα relative to β-actin mRNA levels of 3 independent experiments ± SEM. Paired T test was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

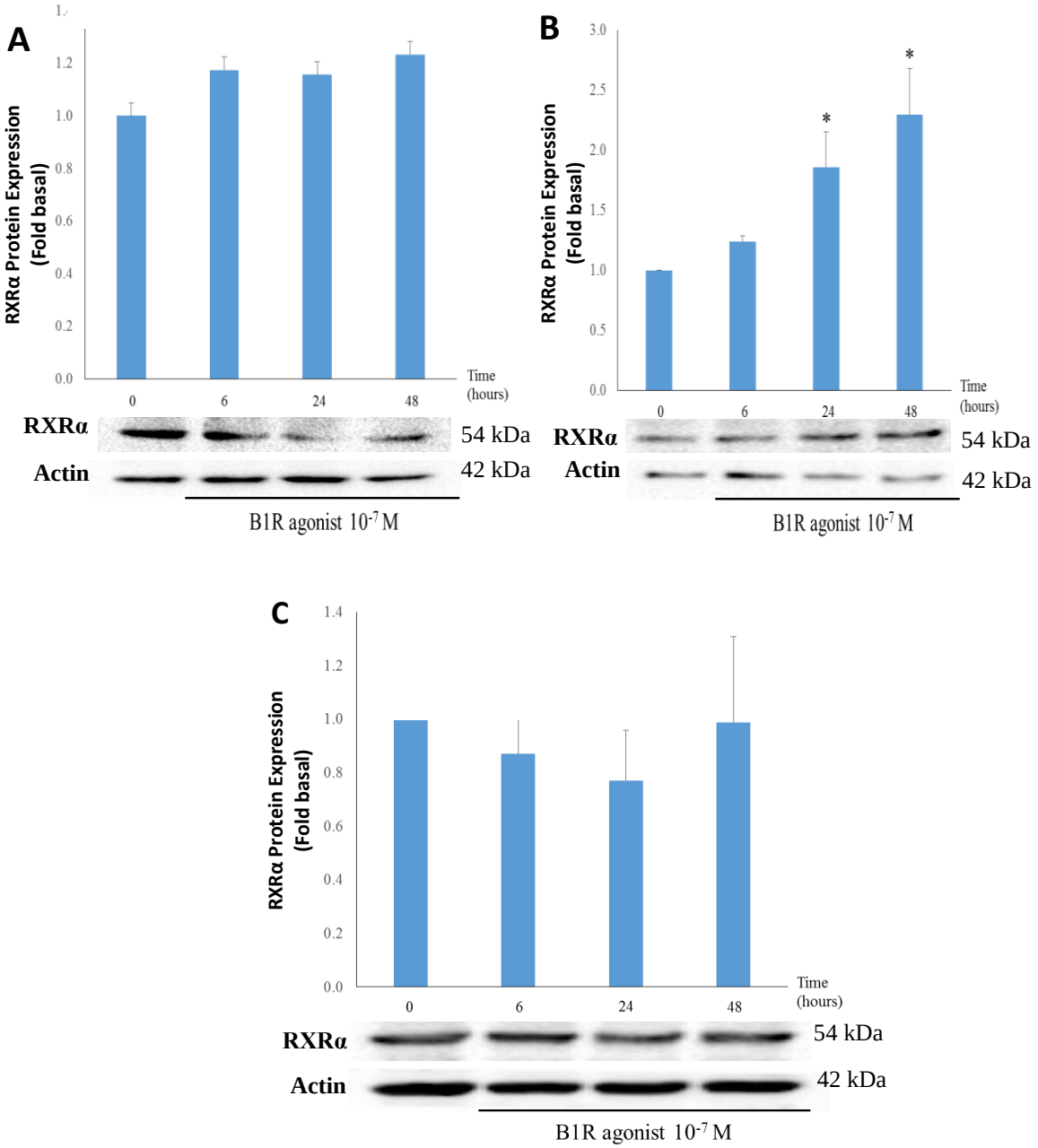

Figure 18. Regulation of RXRα protein levels by B1R agonist in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with B1R agonist [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against RXRα and actin antibodies, RXRα and actin levels were measured by western blot analysis. The bar graph represents the fold change of RXRα relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. Paired T test was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

We wanted to investigate the effects of B2R agonist treatment on gene expression of B1R and B2R and retinoid receptors (RARγ, RARα and RXRα). We used bradykinin as high affinity B2R agonist throughout these experiments.

G. Regulation of Bradykinin 1 Receptor by Bradykinin Treatment in Colorectal Cancer Cells

To investigate the effect of Bradykinin on transcript levels, we measured the mRNA in HCT116, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$ cells, after treatment with bradykinin for 6, 24, and 48 hours as shown in Figure 18. Bradykinin induced the gene expression of B1R at 48 hours of treatment in the HCT116 cells (8 folds ± 2.1, Bradykinin *versus* control $*P<0.05$) (Figure 18A). Furthermore, bradykinin induced B1R transcripts at 6 and 48 hours in the HCT116 $p53^{-/-}$ cell line (23 folds ± 8 and 20.21 folds ± 10 respectively, Bradykinin *versus* control $*P<0.05$) (Figure 18B). However, at 24 and 48 hours of bradykinin treatment, B1R mRNA transcript levels were significantly decreased (0.48 folds ± 0.3 and 0.05 folds ± 0.2 Bradykinin *versus* control $*P<0.05$) (Figure 18C).

Furthermore, the protein levels of B1R were measured after treatment with bradykinin for 6, 24, and 48 hours and presented in Figure19. Bradykinin treatment of HCT116 (Figure 19A) and HCT116 $p21^{-/-}$ cells (Figure 19C) did not significantly alter the levels of B1R proteins. However, bradykinin was shown to significantly decrease the protein expression levels of B1R at 6, 24, and 48 hours of treatment in HCT116 $p53^{-/-}$ cells (0.6 folds ± 0.2, 0.6 folds ± 0.1 and 0.4 folds ± 0.1 respectively, Bradykinin *versus* control $*P<0.05$) (Figure 19B).

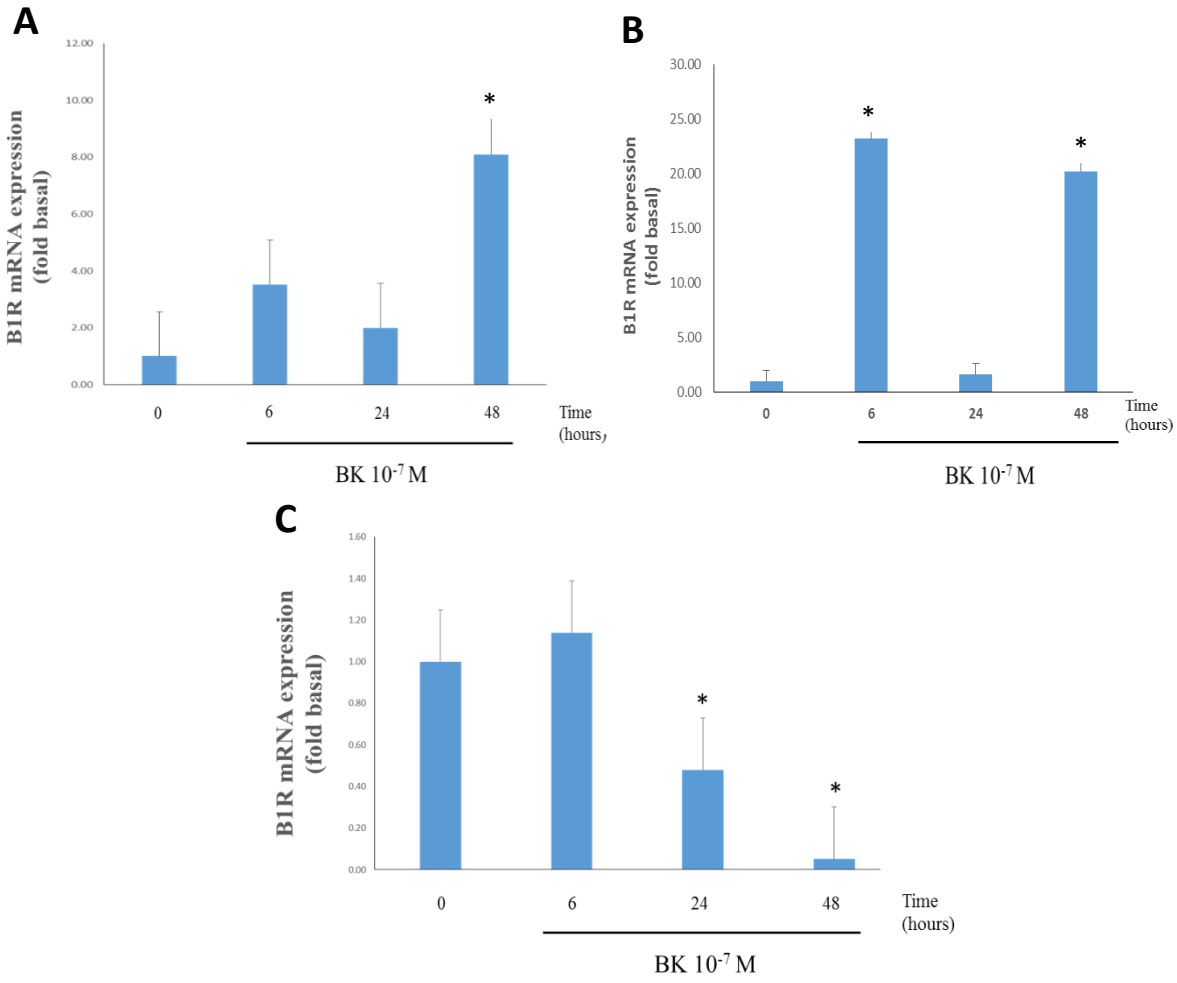

Figure 19. Regulation of B1R transcripts by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby B1R and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents the fold change of B1R relative to β-actin mRNA levels of 3 independent experiments ± SEM. Non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was used to check for the statistical significance in the experiments with *$P<0.05$ *versus* control.

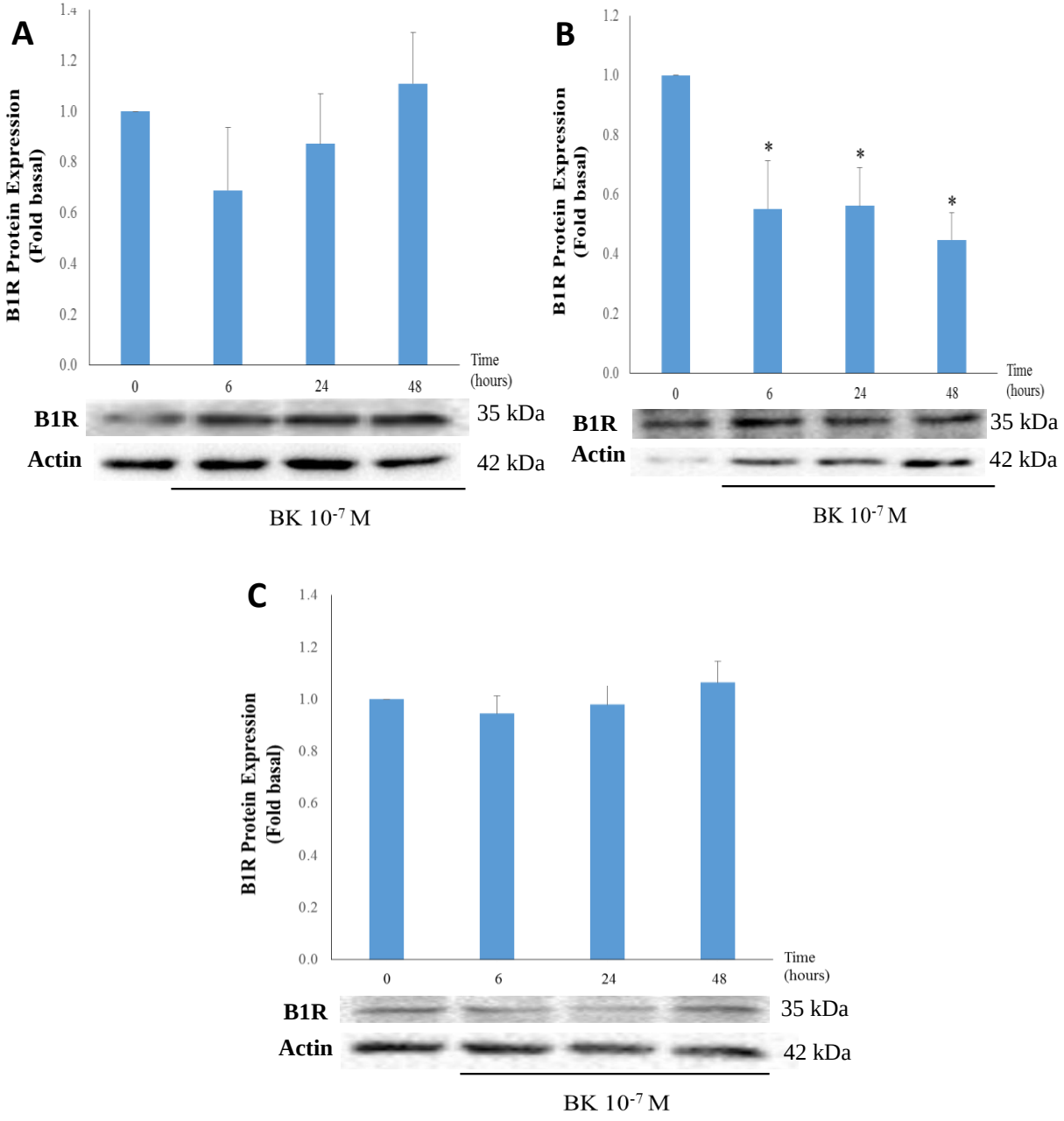

Figure 20. Regulation of B1R protein levels by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against B1R and actin antibodies, B1R and actin levels were measured by western blot analysis. The bar graph represents the fold change of B1R relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. One way ANOVA was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

H. Effect of Bradykinin on Bradykinin 2 Receptor Gene Expression in Colorectal Cancer Cells

The effect of bradykinin on the mRNA levels of B2R is shown in Figure 20. Treatment of HCT116 cells with bradykinin led to decreased levels of the B2R transcripts at 6, 24, and 48 hours (0.5 folds ± 0.2, 0.42 folds ± 0.2 and 0.61 folds ± 0.2 respectively, Bradykinin *versus* control) however, with no statistical significance (Figure 20A). However, bradykinin induced B2R transcript levels at 48 hours of treatment in HCT116 $p53^{-/-}$ cells by 54.45 folds ± 0.2, Bradykinin *versus* control $*P<0.05$) (Figure 20B). Finally, in HCT116 $p21^{-/-}$ cells, bradykinin treatment did not alter significantly B2R transcript levels at 6, and 24 hours of incubation (4.38 folds ± 1 and 3.44 folds ± 1 respectively, Bradykinin *versus* control (Figure 20C).

The effect of Bradykinin on B2R protein levels is presented in Figure 21. The protein levels of B2R were measured after treatment of the different colorectal cancer cells with bradykinin [10^{-7} M] for 6, 24, and 48 hours. Bradykinin treated HCT116 cells showed a significant increase in B2R protein levels at 24 and 48 hours (3.3 folds ± 2.1 and 3.5 folds ± 1.1 respectively, Bradykinin *versus* control $*P<0.05$) (Figure 21A). As for the HCT116 $p53^{-/-}$ cell line, the protein levels of B2R upon bradykinin treatment were increased at 24, and 48 hours (2.4 folds ± 1 and 2.5 folds ± 0.9 respectively, Bradykinin *versus* control $*P<0.05$) (Figure 21B). However, the protein levels of B2R in the HCT116 $p21^{-/-}$ cell line were not significantly changed upon bradykinin treatment at 6, 24, and 48 hours (1.1 folds ± 0.2, 0.9 folds ± 0.2, and 1.5 folds ± 0.3 respectively, Bradykinin *versus* control) (Figure 21C).

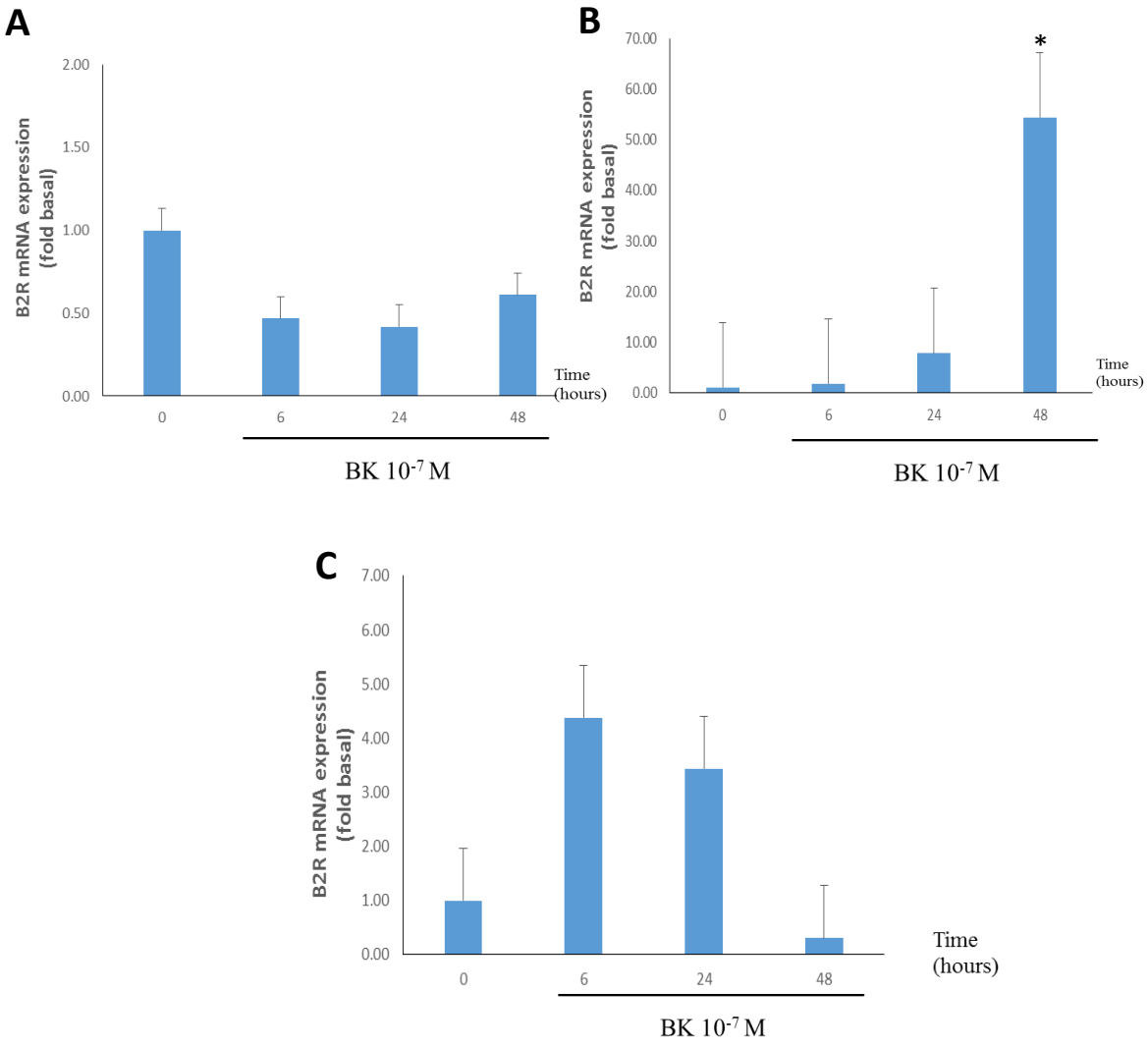

Figure 21. Regulation of B2R transcripts by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby B2R and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents the fold change of B2R relative to β-actin mRNA levels of 3 independent experiments ± SEM. Non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

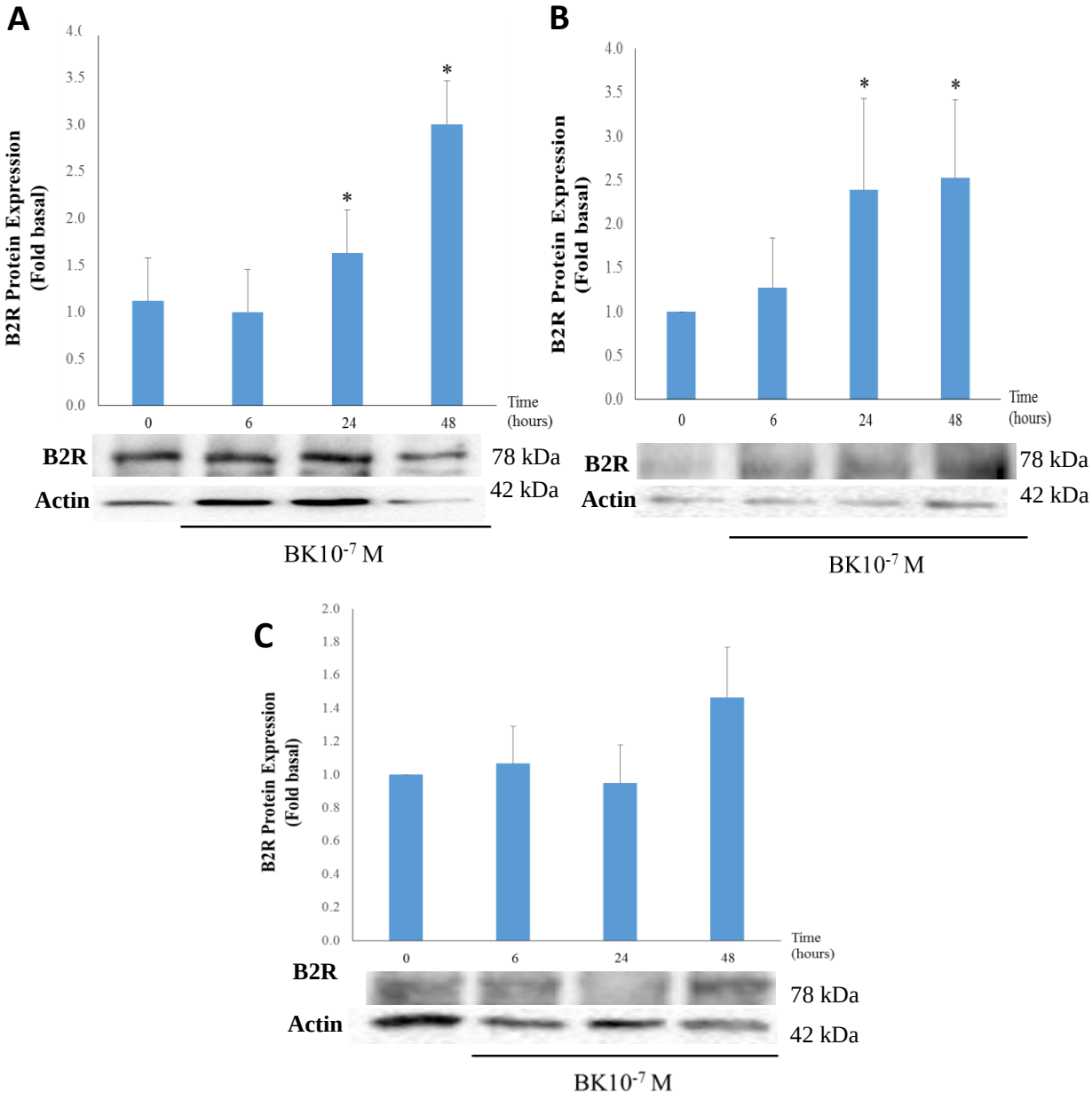

Figure 22. Regulation of B2R protein levels by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against B2R and actin antibodies, B2R and actin levels were measured by western blot analysis. The bar graph represents the fold change of B2R relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. One way ANOVA (A) and Paired T test (B) were used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

I. Effect of Bradykinin on Retinoic Acid Receptor gamma Gene Expression in Colorectal Cancer Cells

The effect of bradykinin on the mRNAs of RARγ is shown in Figure 22. Bradykinin induced the expression of RARγ mRNA levels at 6, and 48 hours of treatment in HCT116 cells (1.55 folds ± 1.1 and 4.48 folds ± 1.2 respectively, Bradykinin *versus* control, with no statistical significance) and reduced its mRNA levels at 24 hours of incubation (0.04 folds ± 0.02, Bradykinin *versus* control, with no statistical significance) (Figure 22A). As for HCT116 $p53^{-/-}$ cell line, bradykinin induced the expression of RARγ at 6, 24, and 8 hours of incubation (4.41 folds ± 1.5, 1.22 folds ± 1.1 and 2.12 folds ± 1 respectively, Bradykinin *versus* control, with no statistical significance) (Figure 22B). However, in HCT116 $p21^{-/-}$ cells, bradykinin was found to potently induce RARγ mRNA at 24 hours (44.1 folds ± 1, Bradykinin *versus* control ***$P<0.001$) (Figure 22C).

Furthermore, the effect of bradykinin on RARγ protein levels was assessed as shown in Figure 23. Bradykinin induced the protein expression of RARγ at 6, 24, and 48 hours of incubation (1.4 folds ± 0.3, 1.5 folds ± 0.2 and 1.7 folds ± 0.7 respectively, Bradykinin *versus* control *$P<0.05$), however it was only significant at 48 hours (Figure 23A). As for the HCT1116 $p53^{-/-}$ cell line, RARγ protein levels were maintained at 6 hours of treatment (1 fold ± 0.1, Bradykinin *versus* control, with no statistical significance) and were decreased at 24 and 48 hours (0.9 folds ± 0.3 and 0.5 folds ± 0.2 respectively, Bradykinin *versus* control *$p<0.05$) (Figure 23B). In HCT116 $p21^{-/-}$ cells, bradykinin induced significantly the expression of RARγ proteins at 48 hours of treatment (2.2 folds ± 0.4 respectively, Bradykinin *versus* control *$P<0.05$) (Figure 23C).

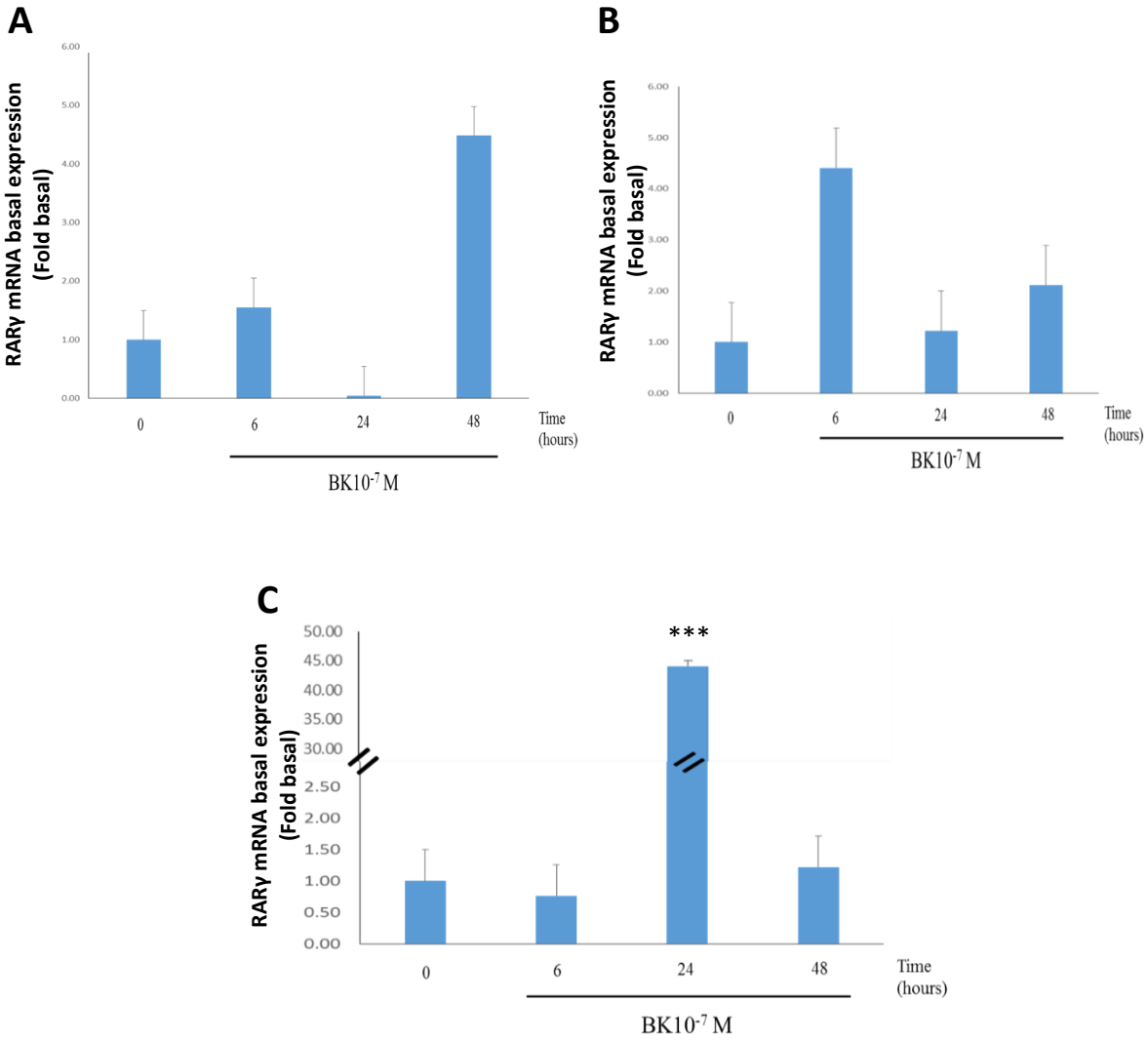

Figure 23. Regulation of RARγ transcripts by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby RARγ and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents the fold change of RARγ relative to β-actin mRNA levels of 3 independent experiments ± SEM. Paired T test was used to check for the

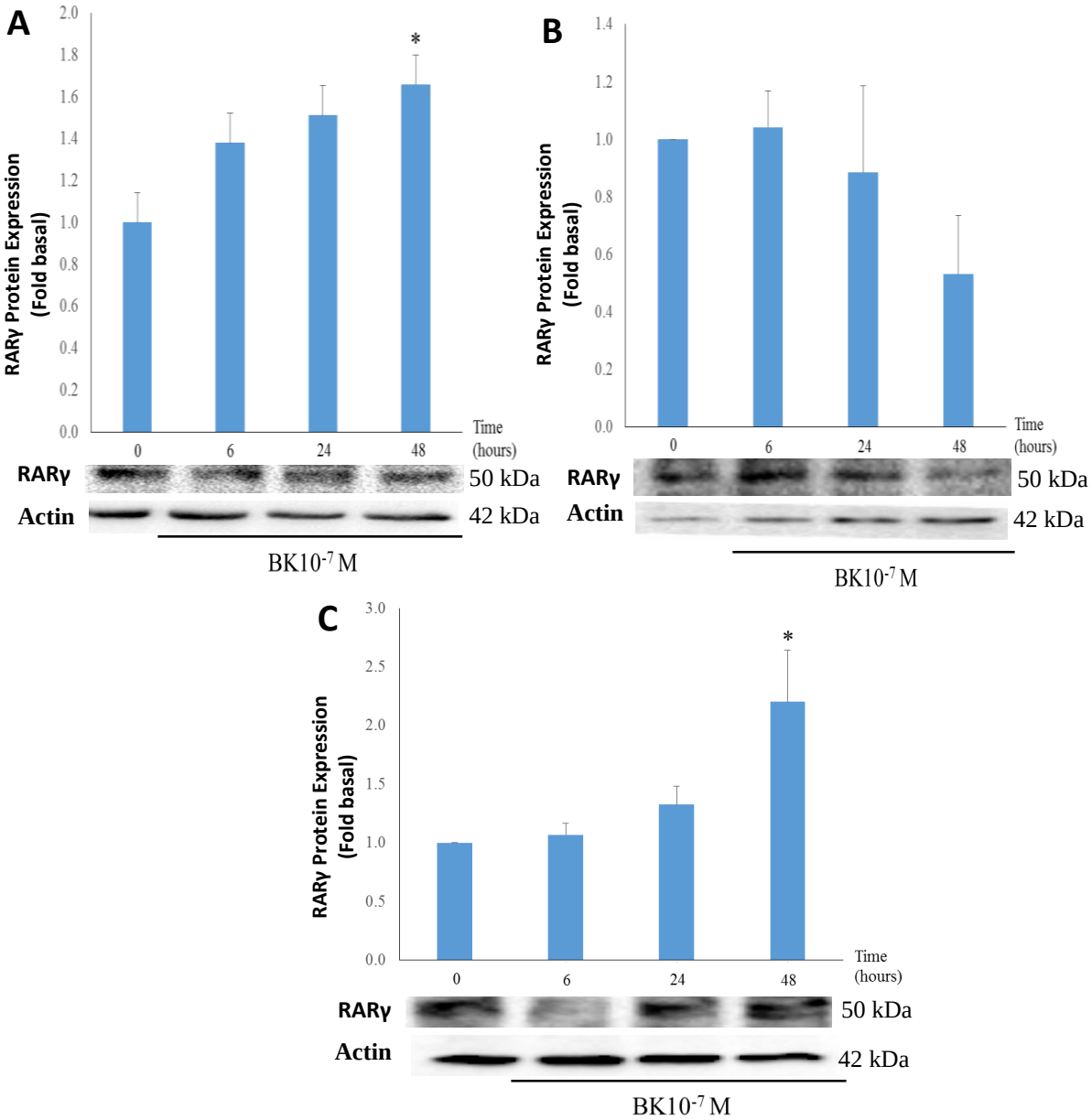

Figure 24. Regulation of RARγ protein levels by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against RARγ and actin antibodies, RARγ and actin levels were measured by western blot analysis. The bar graph represents the fold change of RARγ relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. One way ANOVA (A) and Student-Newman-Keuls Method (C) were used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

J. Effect of Bradykinin on Retinoic Acid Receptor alpha Gene Expression in Colorectal Cancer Cells

The effect of Bradykinin on the mRNA levels of RARα is shown in Figure 24. Bradykinin treatment of HCT116 cells at 6, 24, and 48 hours did not significantly alter RARα transcripts (Figure 24A). However, bradykinin was found to reduce significantly RARα transcripts at 24 hours of treatment (57.85 folds ± 1 and 4.25 ± 1 folds, Bradykinin *versus* control ***P<0.001) (Figure 24B). Finally, in HCT116 $p21^{-/-}$ cells, RARα mRNA levels were not significantly altered at 6, 24, and 48 hours by bradykinin treatment (1.63 folds ± 1.15, 1.98 folds ± 1, and 0.72 folds ± 1 respectively, Bradykinin *versus* control, with no statistical significance) (Figure 24C).

In addition, the effect of bradykinin was also examined on RARα protein levels (Figure 25). Bradykinin treatment did not significantly affect RARα protein levels in the different colorectal cancer cells at the different time points (Figure 25).

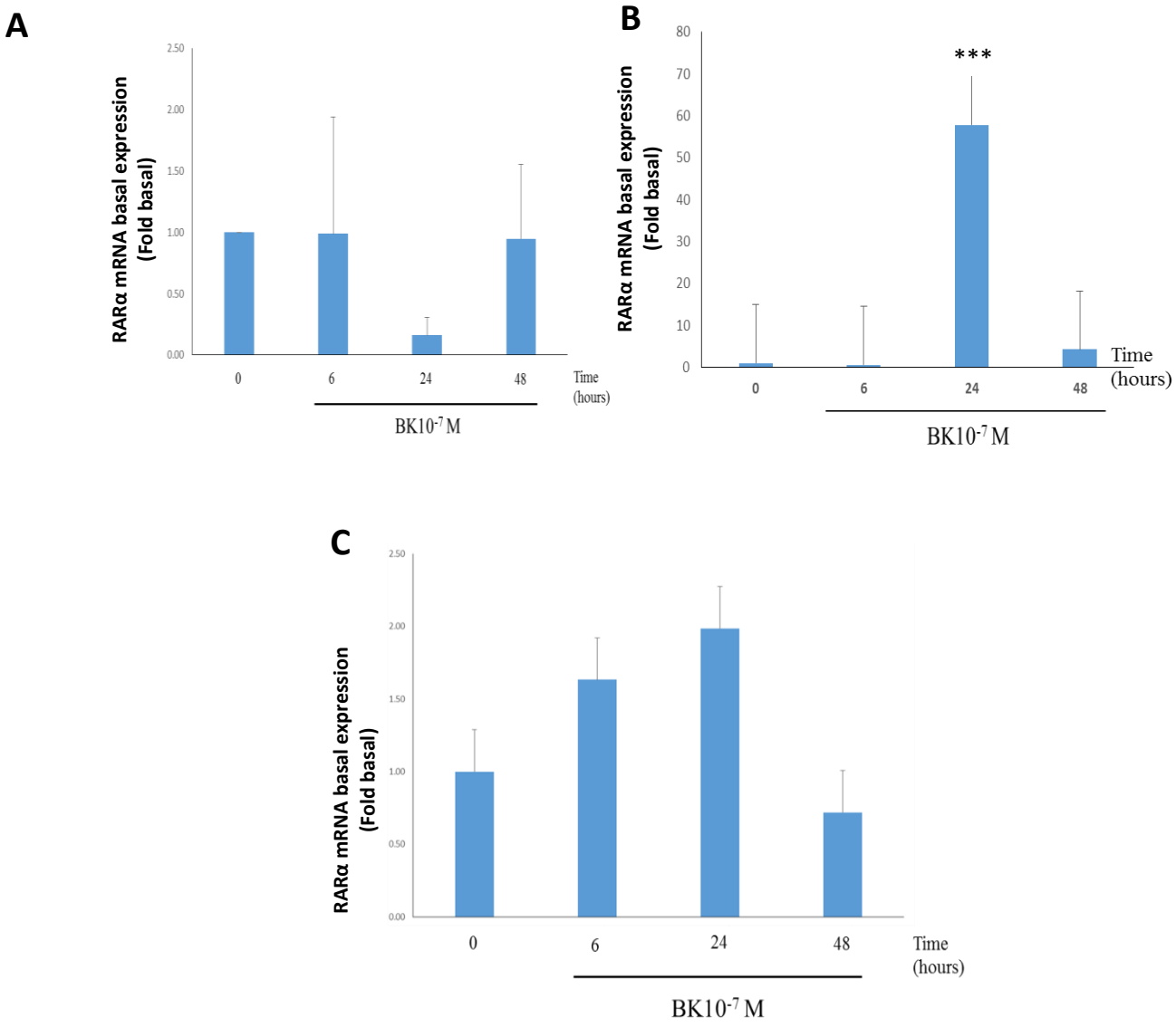

Figure 25. Regulation of RARα transcripts by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby RARα and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents the fold change of RARα relative to β-actin mRNA levels of 3 independent experiments ± SEM. Non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks was used to check for the statistical significance in the experiments with ***P<0.001 *versus* control.

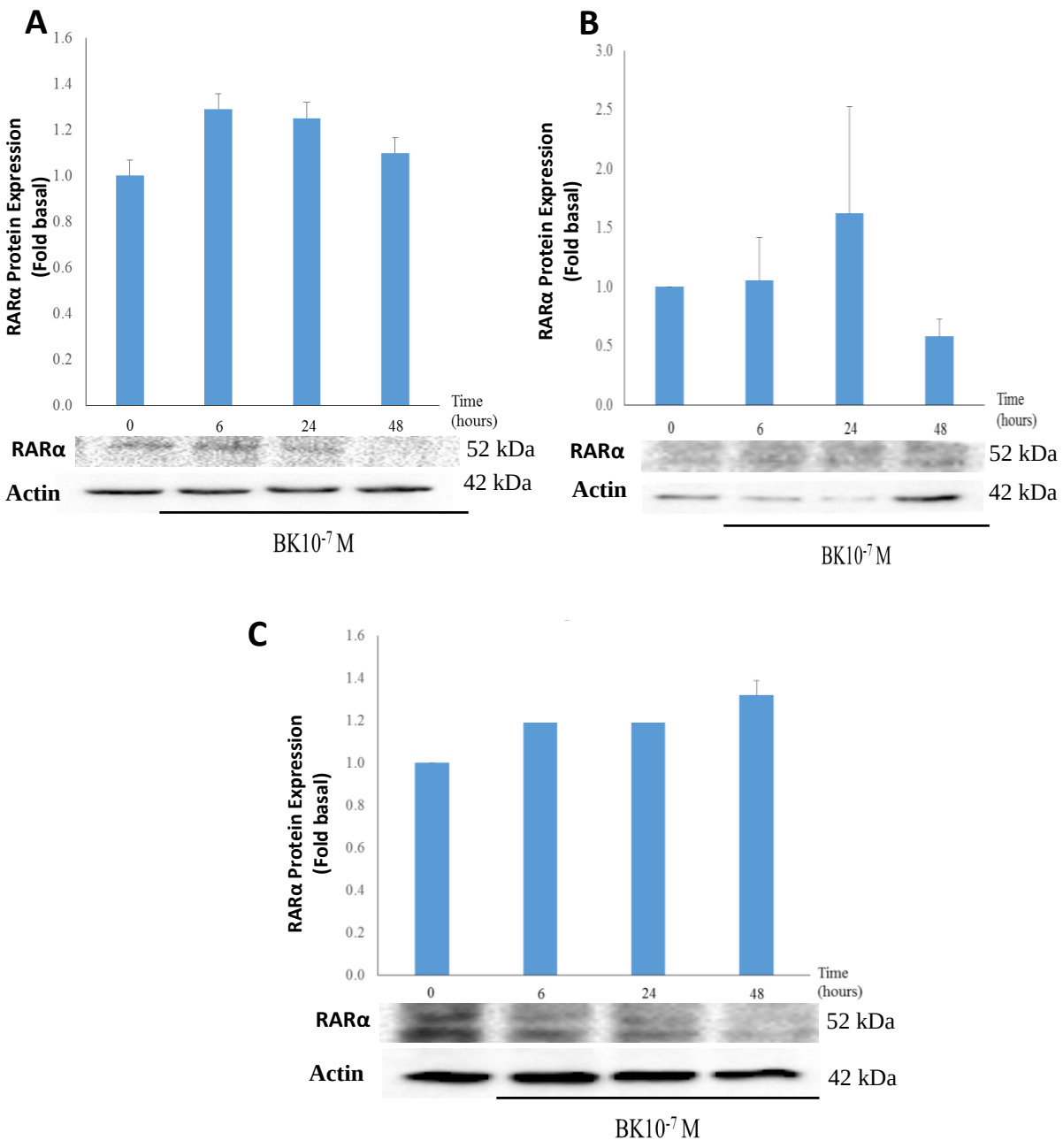

Figure 26. Regulation of RARα protein levels by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against RARα and actin antibodies, RARα and actin levels were measured by western blot analysis. The bar graph represents the fold change of RAR alpha relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments.

K. Effect of Bradykinin on Retinoid X Receptor alpha Gene Expression in Colorectal Cancer Cells

RARs perform their biological functions by heterodimerizing to RXRs. Therefore, we sought to investigate the effects of bradykinin on RXRα gene expression. The effect of bradykinin on the mRNA levels of RXRα is shown in Figure 26. Bradykinin was found to induce the expression of RXRα gene expression at 6, 24, and 48 hours of the HCT116 cells (2 folds ± 1.8, 6.34 folds ± 2 and 1.88 folds ± 1.5 respectively, Bradykinin *versus* control, however, the change was not statistically significant (Figure 26A). Similarly, bradykinin regulation of RXRα at 6, and 48 hours in HCT116 $p53^{-/-}$ cells was not statistically significant (1.2 folds ± 0.8 and 1.82 folds ± 1.2 respectively, Bradykinin *versus* control) however, was decreased at 24 hours of treatment (0.6 folds ± 0.4, Bradykinin *versus* control) (Figure 26B). Finally, bradykinin induced the expression of RXRα at 6, 24, and 48 hours of treatment in HCT116 $p21^{-/-}$ cells but was also found to be no statistical significance (1.34 folds ± 0.68, 9.5 folds ± 1.8 and 5.1 folds ± 1.7 respectively, Bradykinin *versus* control) (Figure 26C).

The effect of bradykinin in colorectal cancer cells was also assessed for RXRα protein levels as shown in Figure 27. Bradykinin was found to induce the expression of RXRα at 6, and 24 hours (1.12 folds ± 0.2 and 1.1 folds ± 0.2 respectively, Bradykinin *versus* control, with no statistical significance) and decreased RXRα mRNA levels at 48 hours in HCT116 cell line (0.9 folds ± 0.4, Bradykinin *versus* control, with no statistical significance) (Figure 27A). Similarly, bradykinin did not regulate RXRα protein levels in HCT116 $p53^{-/-}$ cells (Figure 27B) as well as in HCT116 $p21^{-/-}$ cells except at 48 hours (2.1 folds ± 0.2 respectively, Bradykinin *versus* control *$p<0.05$) (Figure 27C).

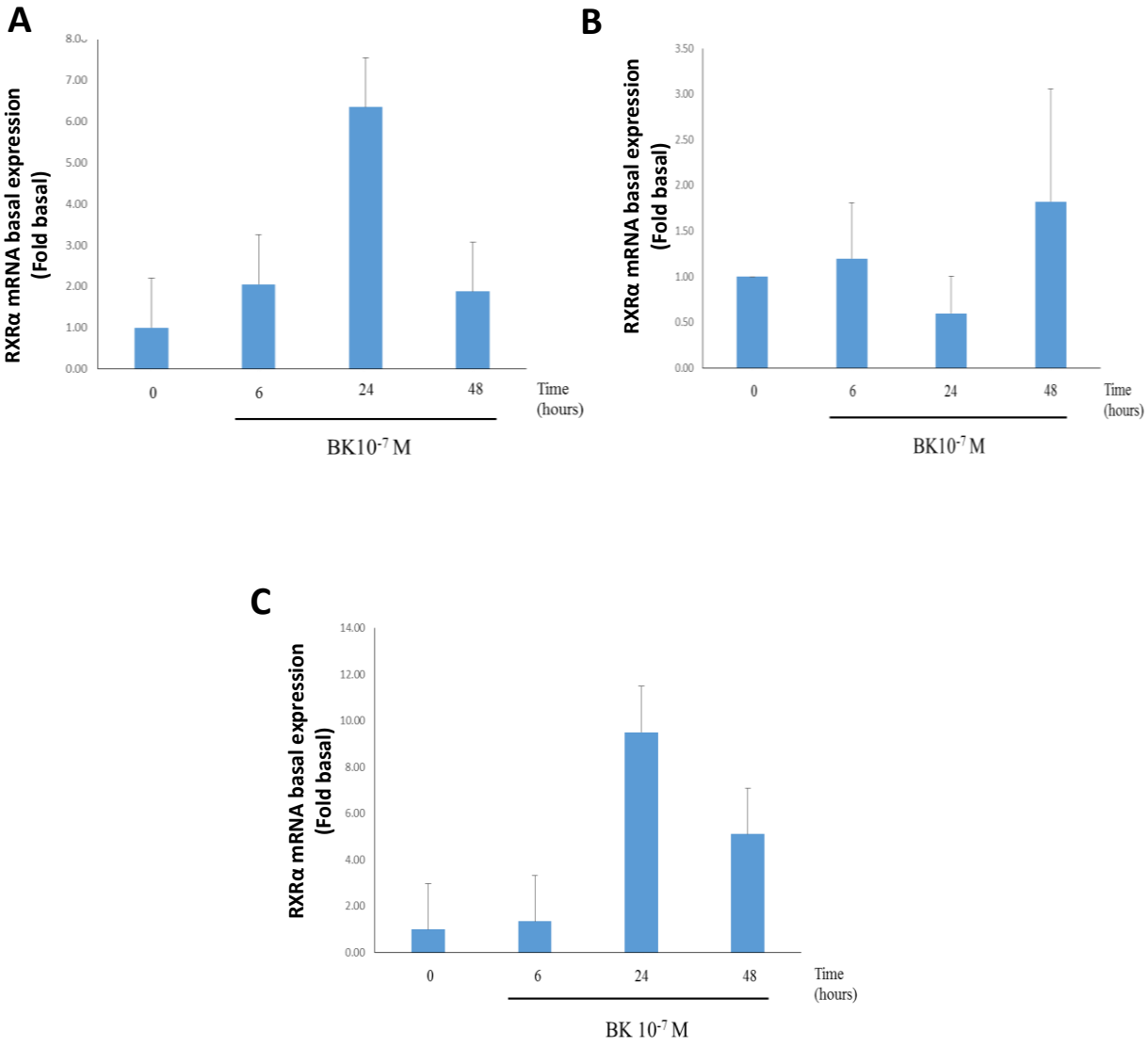

Figure 27. Regulation of RXRα transcripts by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. mRNA was extracted from cells whereby RXRα and β-actin mRNA levels were measured by RT-qPCR. The bar graph represents the fold change of RXRα relative to β-actin mRNA levels of 3 independent experiments ± SEM.

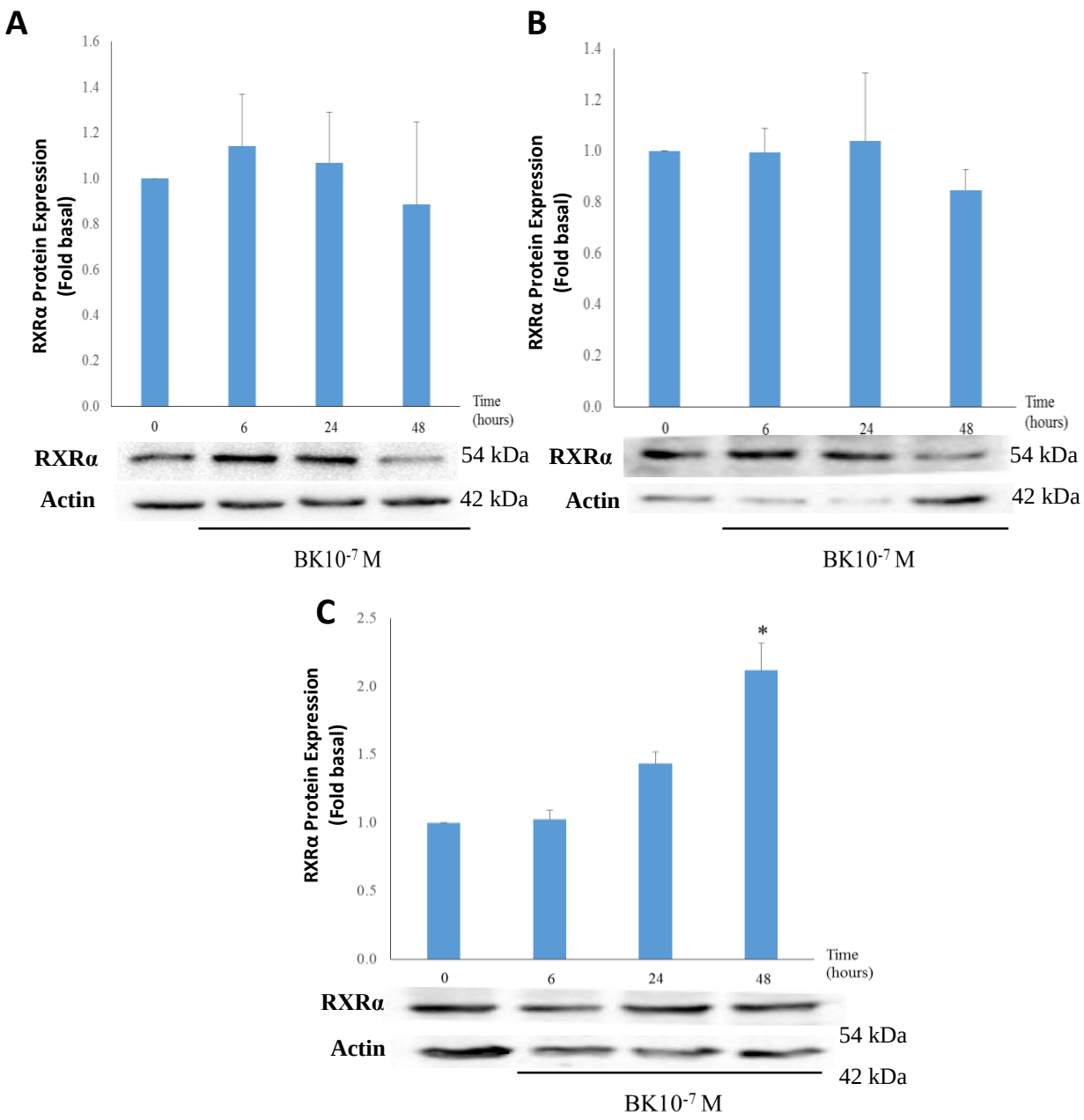

Figure 28. Regulation of RXRα protein levels by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 6, 24, and 48 hours. Total proteins were extracted from cells and immunoblotted against RXRα and actin antibodies, RXRα and actin levels were measured by western blot analysis. The bar graph represents the fold change of RXRα relative to actin protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. Kruskal Wallis (C) was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

Protein kinase B, namely AKT, and ERK1/2 are known to regulate many cellular processes such as cell survival, migration, and proliferation among others. Their activation and/or inactivation could lead to the disruption of multiple crucial mechanisms and thus disease formation. Their signaling, alone or along with other genetic alterations, has been implicated in several malignancies. Moreover, bradykinin is known to induce the phosphorylation of downstream signaling proteins two of which are AKT and ERK1/2 however this was not investigated in colorectal cancer cells.

L. Regulation of Protein Kinase B by Bradykinin in Colorectal Cancer Cells

The activity of AKT was evaluated in three colorectal cancer cell lines in response to treatment with bradykinin. Thus, the effect of bradykinin on AKT phosphorylation status at 5, 10, 30, and 60 minutes was investigated and presented in Figure 28. Bradykinin did not induce the phosphorylation of AKT at any time point of treatment in HCT116 cells, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells (Figure 28). On the contrary, there was a significant decrease in P-AKT upon bradykinin treatment in HCT116 cells at 60 minutes (0.3 folds ± 0.1 folds, Bradykinin *versus* control $*P<0.05$) (Figure 28A) and at 10 minutes and 60 minutes in HCT116 p53-/- cells (0.4 folds ± 0, and 0.3 folds ± 0 respectively, Bradykinin *versus* control $*P<0.05$) (Figure 28B). Comparison were done relative to total AKT protein levels.

M. Induction of Extracellular Signal-Regulated Kinase by Bradykinin in Colorectal Cancer Cells

The activity of ERK1/2 was assessed after treatment with bradykinin in the three colorectal cancer cell lines of interest. In HCT116 cells, bradykinin induced the expression of ERK 1/2 at 5, 10, 30, and 60 minutes of treatment (1.1 folds ± 0.4, 1.3 folds ± 0.2, 1.2 folds ± 0.2 and 1.9 folds ± 0.2 respectively, Bradykinin *versus* control $*P<0.05$). Comparison were done relative to total ERK1/2 protein levels (Figure 29A).

As for HCT116 $p53^{-/-}$ cells, bradykinin treatment for 30 and 60 minutes induced the phosphorylation of ERK1/2 (3.4 folds ± 2.4 and 3.5 folds ± 2.3 respectively, Bradykinin *versus* control $**P<0.01$). Comparison were done relative to total ERK1/2 protein levels (Figure 29B).

Similarly, in HCT116 $p21^{-/-}$ cells, bradykinin treatment for 30 and 60 minutes induced the phosphorylation of ERK1/2 (1.6 folds ± 0.4 and 1.6 folds ± 0.2 respectively, Bradykinin *versus* control $*P<0.05$). Comparison were done relative to total ERK1/2 protein levels (Figure 29C).

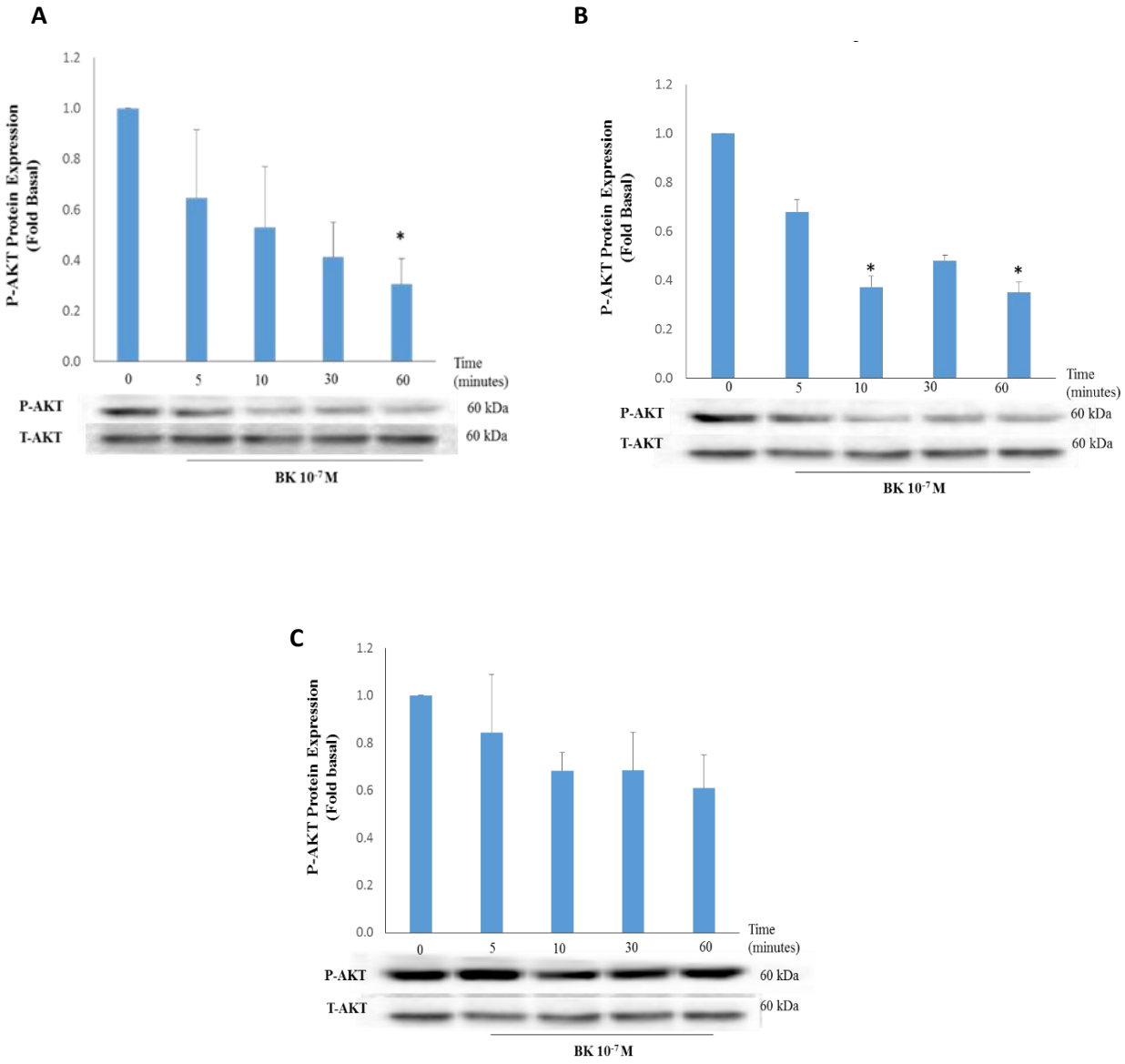

Figure 29. Regulation of AKT activity by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 5, 10, 30, and 60 minutes. Total proteins were extracted from cells and immunoblotted against phosphorylated-AKT (P-AKT) and total-AKT (T-AKT) antibodies. P-AKT and T-AKT levels were measured by western blot analysis. The bar graph represents the fold change of P-AKT relative to T-AKT protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. Mann-Whitney U Test (A and B) was used to check for the statistical significance in the experiments with *P<0.05 *versus* control.

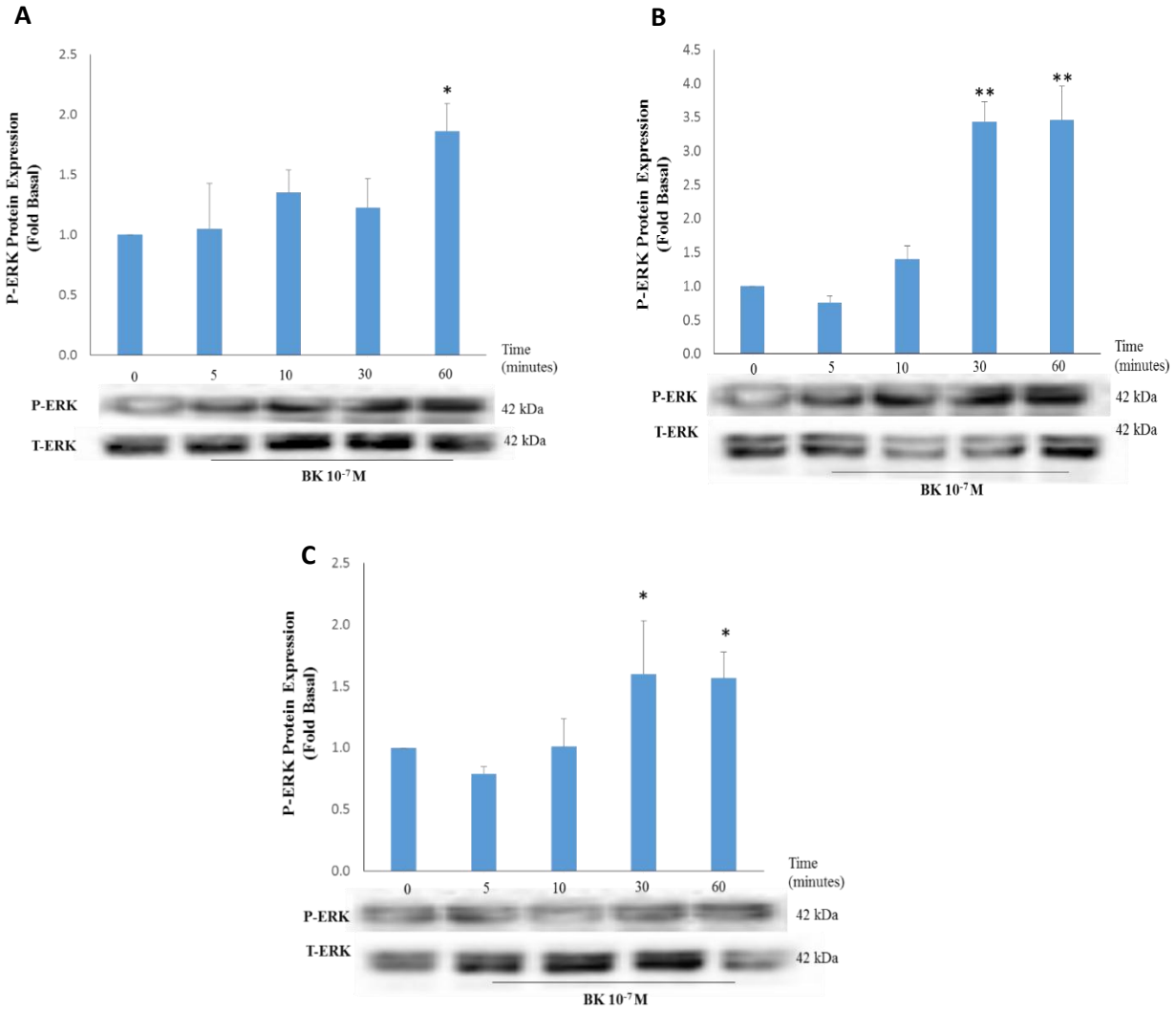

Figure 30. Regulation of ERK1/2 by bradykinin in colorectal cancer cells. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cell lines were treated with bradykinin [10^{-7} M] for 5, 10, 30, and 60 minutes. Total proteins were extracted from cells and immunoblotted against phosphorylated ERK1/2 (P-ERK) and total ERK1/2 (T-ERK) antibodies. P-ERK and T-ERK levels were measured by western blot analysis. The bar graph represents the fold change of P-ERK relative to T-ERK protein levels of 3 independent experiments ± SEM. Western blot analysis is representative of 3 independent experiments. Mann-Whitney U Test (A, B, and C) was used to check for the statistical significance in the experiments with *P<0.05 and **P<0.01 *versus* control.

Three well-characterized human colorectal cancer cell lines with different *p53* and *p21* status (HCT116, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$) were selected to characterize for the effects of bradykinin and B1R agonist on their growth (Figures 30 and 31).

N. Bradykinin Treatment Does not Affect the Growth of Colorectal Cancer Cells

As observed by the nonradioactive cell proliferation MTT growth assay, HCT116 cells were relatively resistant to treatment with bradykinin. We used synthetic retinoid at 10^{-6} M as a positive control to show that the three types of colorectal cancer cell lines are sensitive to its growth suppressive activities (Figure 30). At a concentration of 10^{-6} M as ST1926 inhibited the growth of HCT116 cells by 30% at 24 hours, 54% at 48 hours, and 77% at 72 hours (Figure 30A). Similarly, HCT116 $p53^{-/-}$ cells were relatively resistant up to 10^{-6} M Bradykinin. However, 1 μM ST1926 resulted in a time-dependent growth inhibition in all tested cell lines (Figure 30). Only, HCT166 $p21^{-/-}$ cells showed low sensitivity at 10^{-6} M bradykinin at 48 hours post treatment which caused 25% growth suppression.

O. Bradykinin 1 Receptor Agonist Treatment Does not Affect the Growth of Colorectal Cancer Cells

The human colorectal cancer cell lines were shown by MTT assay to be relatively resistant to treatment with B1R agonist up to 1 μM concentrations (Figures 30 and 31). Cell growth relative to control was found to be 103%, 83%, and 77% in HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells respectively, after 72 hours of treatment with B1R agonist (Figure 31). Based on the above results, we conclude that the three human colorectal cancer cell lines are relatively resistant to Bradykinin, and B1R agonist irrespective of p53, and p21 status.

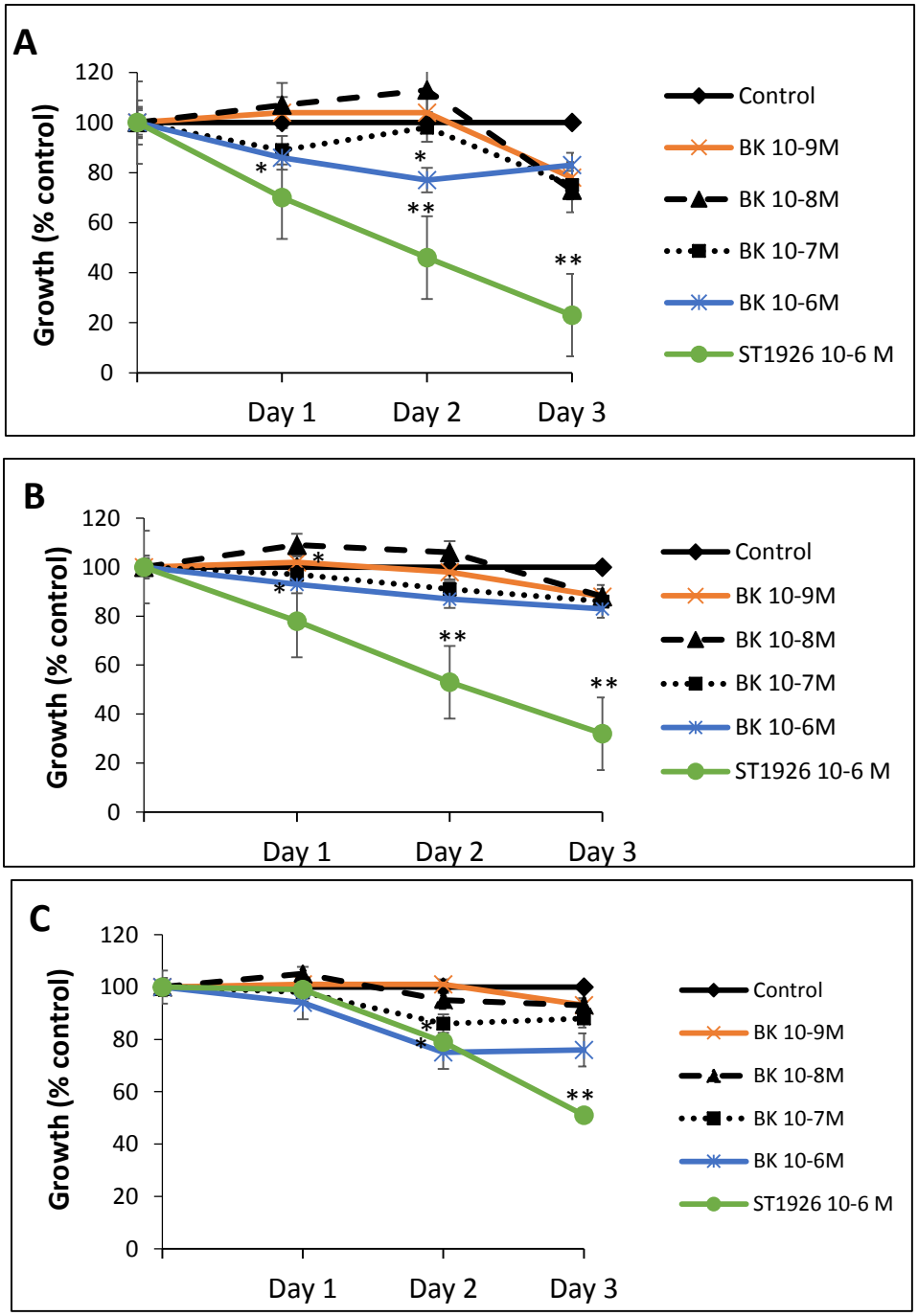

Figure 31. Effect of bradykinin on the growth of human colorectal cancer cell lines. (A) HCT116 (B), HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$. Cells were seeded in 96-well plates and treated with 0.1% DMSO (control) or the indicated concentrations of bradykinin and ST1926. Cell growth was assayed in triplicate wells using the MTT assay. Results are expressed as percentage of control and represent the average of three independent experiments ± SEM. Dunn's test was used to check for statistical significance with *P<0.05 or **P<0.01 *versus* control.

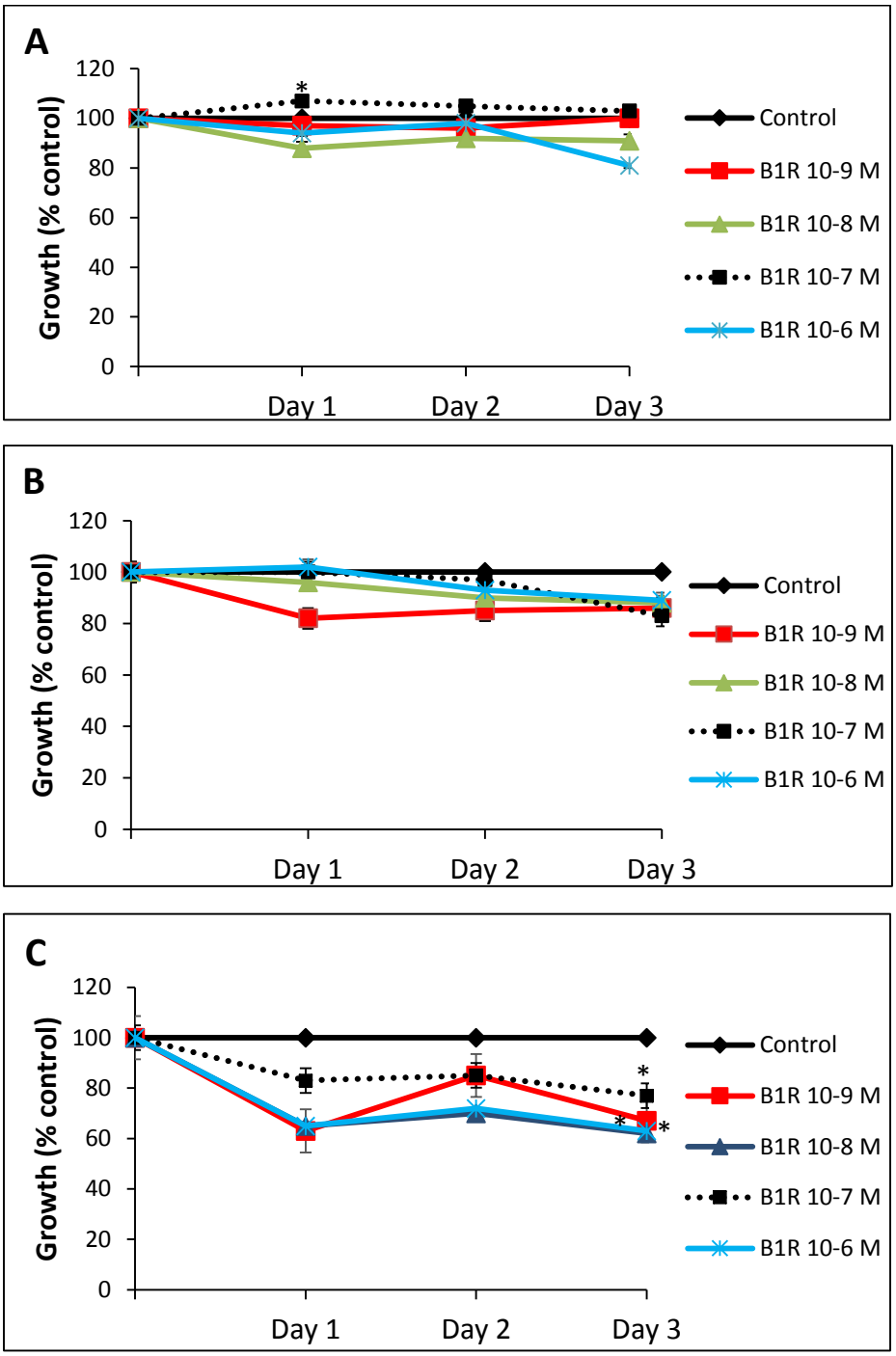

Figure 32. Effect of B1R agonist on the growth of human colorectal cancer cell lines. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$. Cells were seeded in 96-well plates and treated with 0.1% DMSO (control) or the indicated concentrations of B1R agonist. Cell growth was assayed in triplicate wells using the MTT assay. Results are expressed as percentage of control and represent the average of three independent experiments ± SEM. Dunn's test was used to check for statistical significance with *P<0.05 *versus* control.

P. The Effect of Bradykinin on the Subcellular Localization of the Retinoic Acid Receptor gamma

To study the subcellular localization of the RARγ with its possible crosstalk with B2R, immunofluorescence was performed on HCT116, HCT116 $p53^{-/-}$, and HCT116 $p21^{-/-}$ cells under a 24 hour serum starvation, and various bradykinin treatment conditions. All three cell types were starved for 24 hours, and treated with different bradykinin [10^{-7} M] for 30 minutes or 24 hours (Figure 32). In HCT116 cells, the non-stimulated cells starved for 24 hours displayed both a nuclear, and a cytoplasmic localization of RARγ within the cell (Figure 32A). After 24 hours treatment with bradykinin, RARγ seemed to be mainly localized in the nucleus. However, bradykinin treatment for 30 minutes resulted in punctate distribution of RARγ within the cytoplasm (Figure 32A). As for HCT116 $p53^{-/-}$ cell line, the non-stimulated cells presented with RARγ in a diffused cytoplasmic localization with some massive cytoplasmic aggregates. In addition, when treated with bradykinin for 30 minutes, HCT116 $p53^{-/-}$ cells exhibited RARγ punctates in the cytoplasm. Finally, after 24 hour treatment with bradykinin, RARγ became substantially localized within the nucleus, while maintaining some cytoplasmic aggregates that were evident at basal unstimulated conditions (Figure 32B). Finally, in HCT116 $p21^{-/-}$ cell line, the control cells showed a diffused distribution of RARγ within the cell with some massive cytoplasmic aggregates. Furthermore, bradykinin treatment for 30 minutes resulted in massive cytoplasmic distribution of the RARγ. However, treatment with bradykinin for 24 hours, did not have any effect on the distribution of this receptor of interest as compared to the unstimulated HCT116 $p21^{-/-}$ cells (Figure 32C).

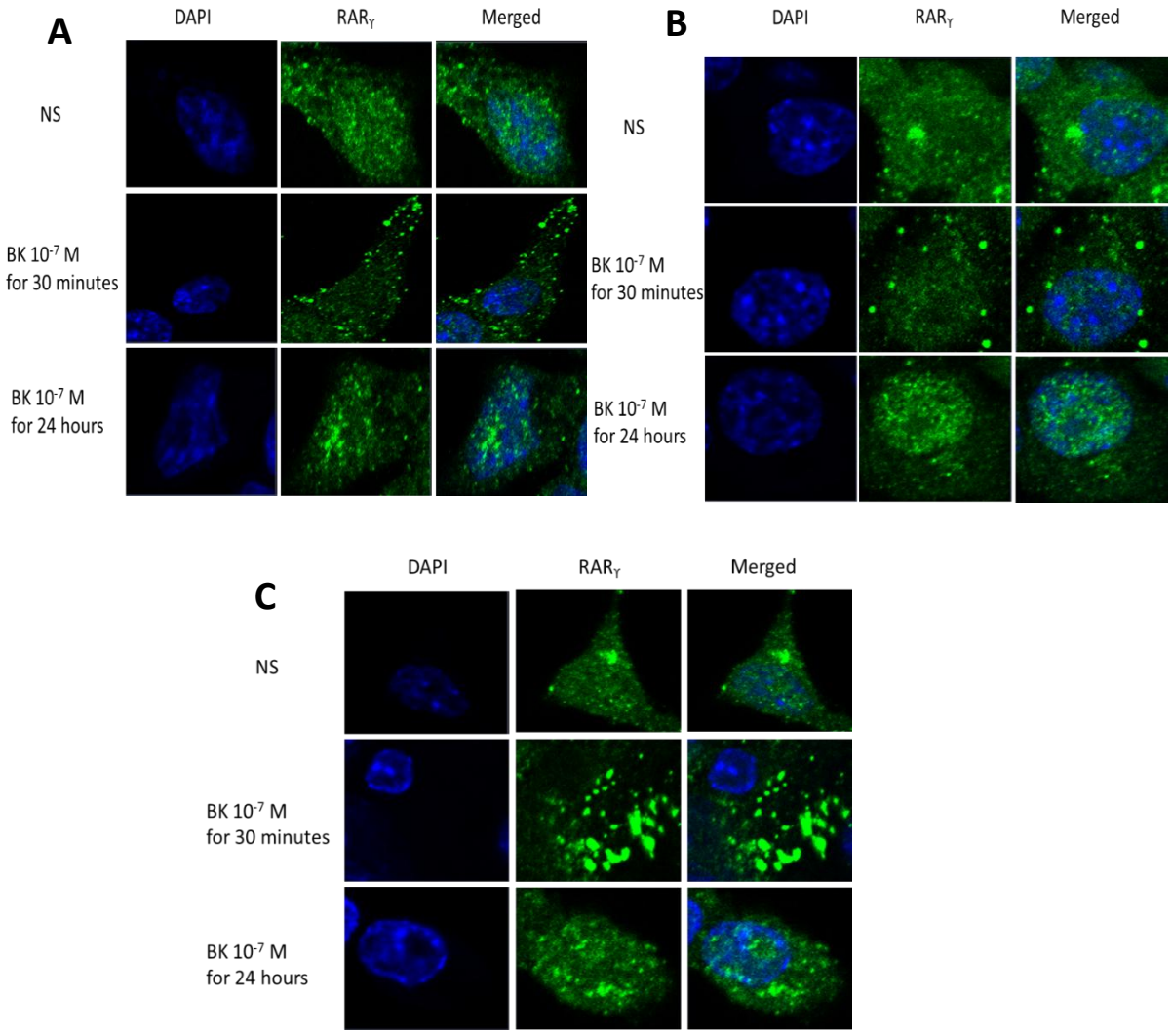

Figure 33. Regulation of intracellular localization of RARγ by bradykinin treatment. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cells were serum starved for 24 hours and either non-stimulated (NS) or treated with bradykinin [10^{-7} M] for 30 minutes or 24 hours. Cells were immunostained using anti-rabbit RARγ antibody and nuclei were counterstained with DAPI. Cells were visualized at a magnification of 63x using confocal microscopy and are representative of several fields.

Q. The Effect of Bradykinin on the Subcellular Localization of the Bradykinin 2 Receptor

Immunofluorescence was employed as to check for the B2R distribution in the three human colorectal cancer cell lines after 24 hour serum starvation conditions (Figure 33). In HCT116 cells, the non-stimulated cells showed a B2R distribution mainly as bundles at the inner part of the cell membranes. Yet, after treatment with bradykinin for 30 minutes, B2R became localized in the cytoplasm and 24 hour bradykinin treatment resulted in the nuclear retention of the B2R (Figure 33A). Regarding the HCT116 $p53^{-/-}$ cells, B2R distribution was diffused in the cytoplasm of the non-stimulated cells with bundles at the inner part of the cell membranes. However, at 30 minutes treatment with bradykinin, B2R was almost entirely cytoplasmic. After the 24 hour treatment with bradykinin, B2R was totally absent from the nucleus, and punctates were noticed in the cytoplasm (Figure 33B). As for the non-stimulated HCT116 $p21^{-/-}$ cells as well as those treated for 24 hours with bradykinin, they showed a diffused cytoplasmic, and nuclear distribution of the B2R with cytoplasmic aggregates. However, bradykinin treatment for 30 minutes led to an exclusive cytoplasmic punctate distribution of this receptor (Figure 33C).

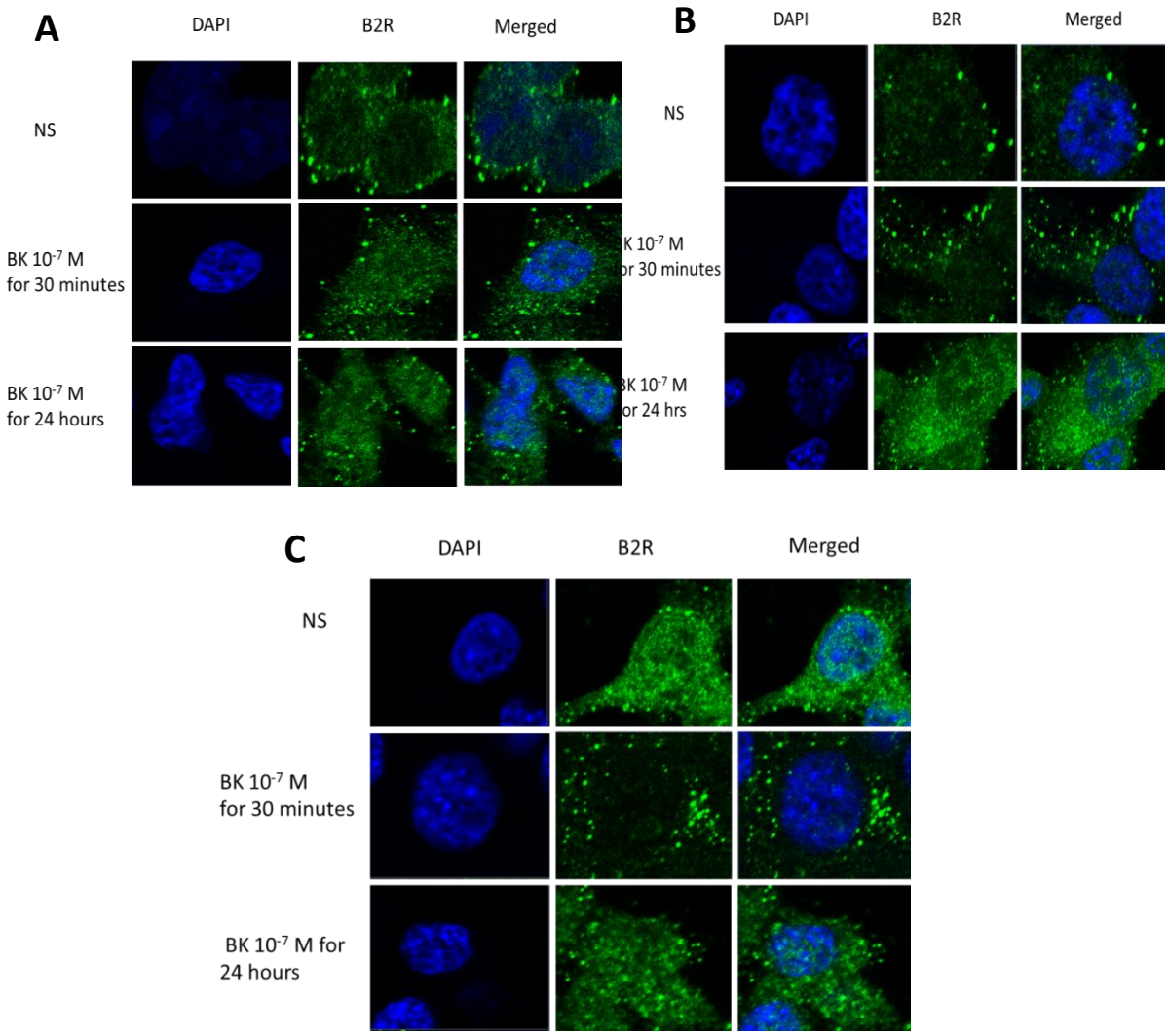

Figure 34. Regulation of intracellular localization of B2R by bradykinin treatment. (A) HCT116, (B) HCT116 $p53^{-/-}$, and (C) HCT116 $p21^{-/-}$ cells were serum starved for 24 hours and either non-stimulated (NS) or treated with bradykinin [10^{-7} M] for 30 minutes or 24 hours. Cells were immunostained using anti-rabbit B2R antibody and nuclei were counterstained with DAPI. Cells were visualized at a magnification of 63x using confocal microscopy and are representative of several fields.

R. Involvement of the Extracellular Signal-Regulated Kinase1/2 on the Retinoic Acid Receptor gamma Subcellular Localization

To check whether the ERK1/2, which is downstream of bradykinin, has a role in the cellular localization of the RARγ in all the tested human colorectal cancer cell lines, immunofluorescence was performed on 30 minutes or 24 hours starved cells and treated with bradykinin [10^{-7} M] and/or the ERK1/2 inhibitor, PD98059 [$25X10^{-6}$ M] (Figures 34, 35, and 36). Figure 34A reveals that in the HCT116 non-stimulated cells starved for 30 minutes, RARγ distribution was diffused. However, after treatment with PD98059, RARγ localization became nuclear. However, stimulation of HCT116 cells with bradykinin for 30 minutes led to the formation of cytoplasmic aggregates. Finally, pretreatment of these cells with PD98059 for 30 minutes prior to stimulation with bradykinin for 30 minutes resulted in both nuclear, and cytoplasmic localization of RARγ (Figure 34A). Following serum starvation for 24 hours, different results were revealed. The HCT116 control cells had a nuclear distribution of the RARγ, yet after PD98059 treatment for 30 minutes it became cytoplasmic (Figure 34B). Likewise, stimulation of these cells with bradykinin for 30 minutes, led to its localization in the cytoplasm. Finally, pretreatment of HCT116 cells with PD98059 for 30 minutes prior to stimulation with bradykinin for 30 minutes, resulted in nuclear localization of RARγ as seen in the case of the HCT116 control cells (Figure 34B).

In HCT116 $p53^{-/-}$ cells starved for 30 minutes, whether the non-stimulated cells or cells treated with PD98059 for 30 minutes, the RARγ localization was nuclear (Figure 35A). However, stimulation of cells with bradykinin for 30 minutes led to its retention within the cytoplasmic compartment. Last but not least, pretreatment of cells with PD98059 for 30

minutes prior to stimulation with bradykinin for 30 minutes returned the RARγ back to the nucleus (Figure 35A). After changing the serum starvation duration for 24 hours, results were different. The unstimulated HCT116 $p53^{-/-}$ cells starved for 24 hours, displayed a cytoplasmic distribution of RARγ. However, treatment with PD98059 alone, bradykinin alone, or pretreatment with PD98059 followed by stimulation with bradykinin, led to a diffused distribution of the RARγ in the cellular cytoplasm, with no nuclear localization (Figure 35B).

The unstimulated HCT116 $p21^{-/-}$ cells starved for 30 minutes, RARγ was mainly localized in the nucleus. However, treatment with PD98059 for 30 minutes, led to the massive distribution of RARγ into the cytoplasmic compartment. Nevertheless, bradykinin treatment for 30 minutes resulted in both nuclear, and cytoplasmic localization of the RARγ. Likewise, cells pretreated with PD98059 for 30 minutes prior to stimulation with bradykinin for another 30 minutes, presented with both nuclear, and cytoplasmic localization of RARγ (Figure 36A). Regarding the HCT116 $p21^{-/-}$ cells starved for 24 hours, in the non-stimulated control, RAR gamma distribution was much diffused with evident cytoplasmic aggregates (Figure 36B). Treatment with PD98059 did not have any effect on RARγ localization. However, stimulation of cells with bradykinin for 30 minutes led to its massive cytoplasmic distribution and aggregation. Pretreatment with PD98059 for 30 minutes prior to stimulation with bradykinin for 30 minutes resulted mainly in nuclear localization of RARγ, with some minimal distribution in the cytoplasmic compartment (Figure 36B).

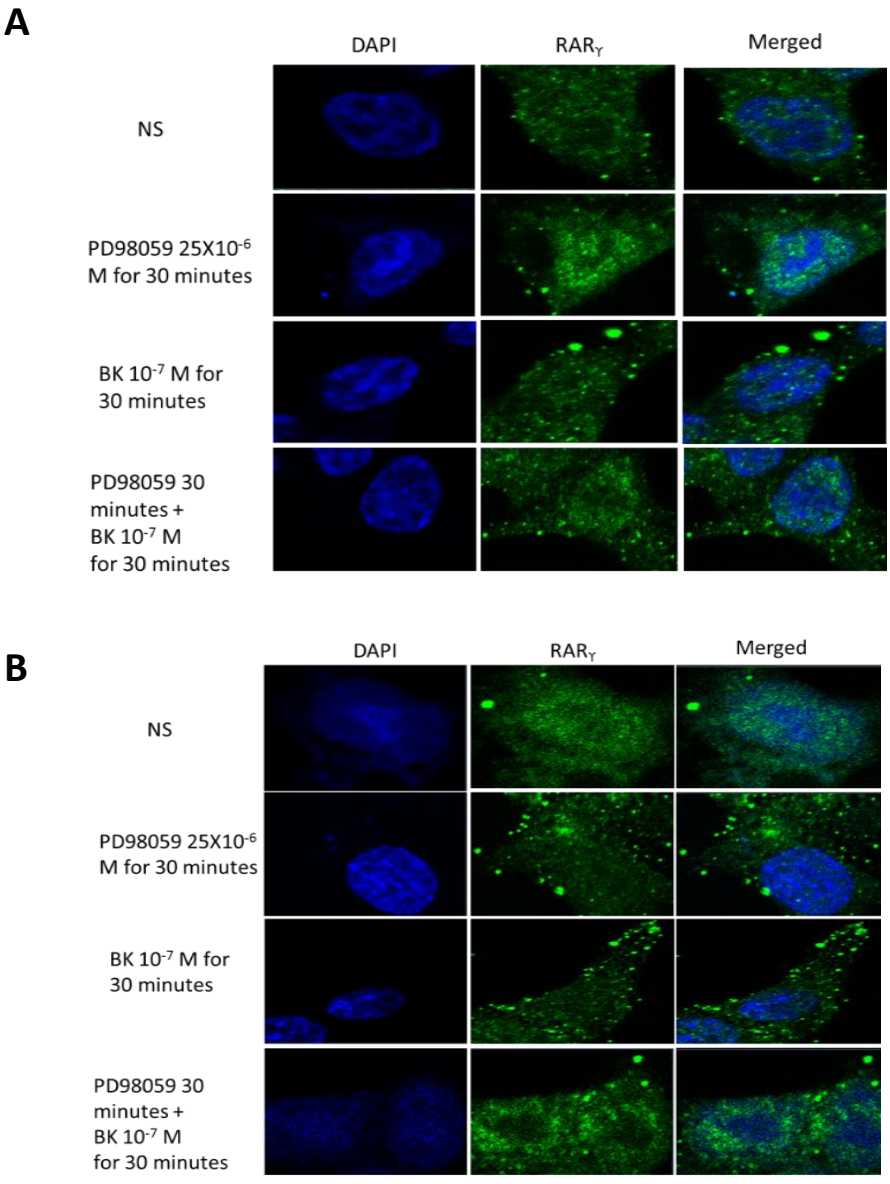

Figure 35. Regulation of intracellular localization of RARγ by serum starvation conditions in HCT116 cells. (A) Starved for 30 minutes (B) Starved for 24 hours, and treated with bradykinin [10^{-7} M] or/and PD98059 [$25X10^{-6}$ M] for 30 minutes. Cells were immunostained using anti-rabbit RARγ antibody and nuclei were counterstained with DAPI. Cells were visualized at a magnification of 63x using confocal microscopy and are representative of several fields.

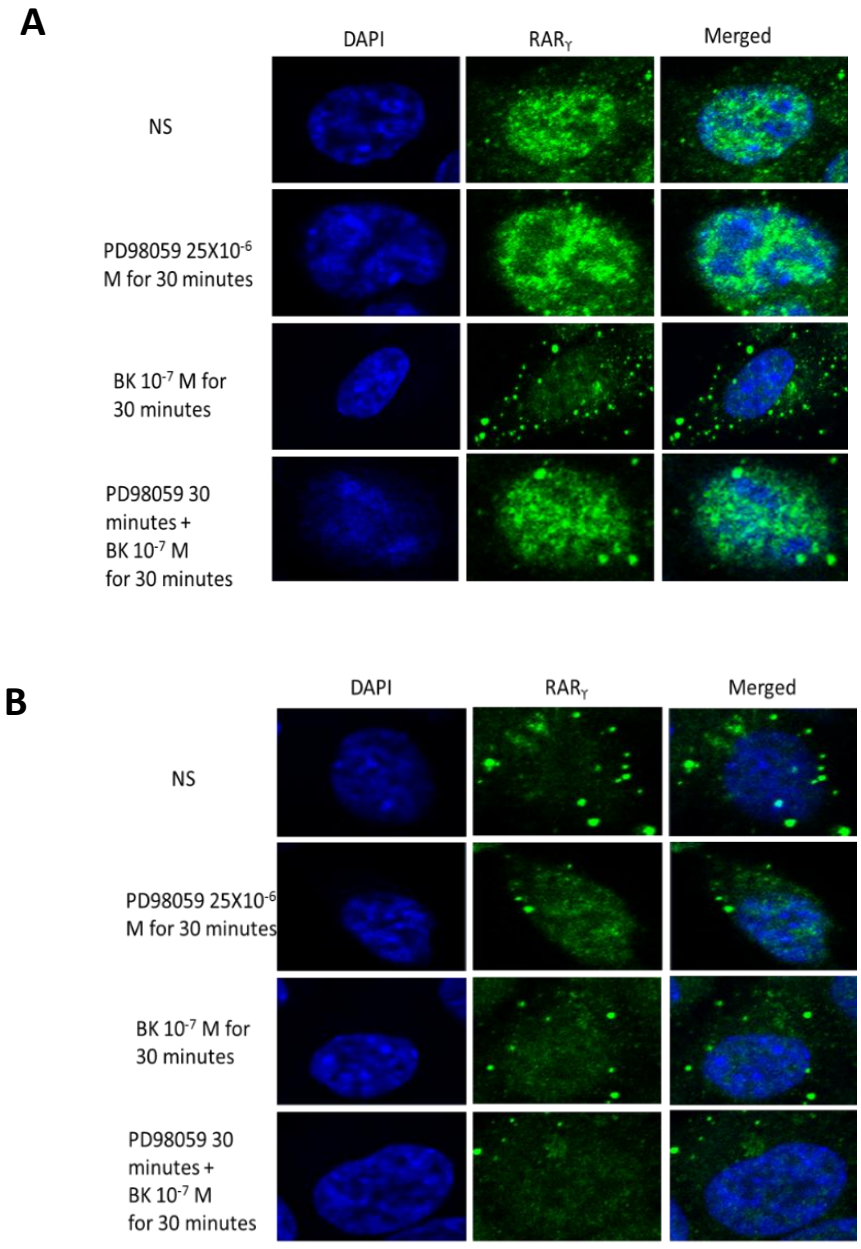

Figure 36. Regulation of intracellular localization of RARγ by serum starvation conditions in HCT116 p53-/- cells. (A) Starved for 30 minutes (B) Starved for 24 hours, and treated with bradykinin [10^{-7}M] or/and PD98059 [$25X10^{-6}$ M] for 30 minutes. Cells were immunostained using anti-rabbit RARγ antibody and nuclei were counterstained with DAPI. Cells were visualized at a magnification of 63x using confocal microscopy and are representative of several fields.

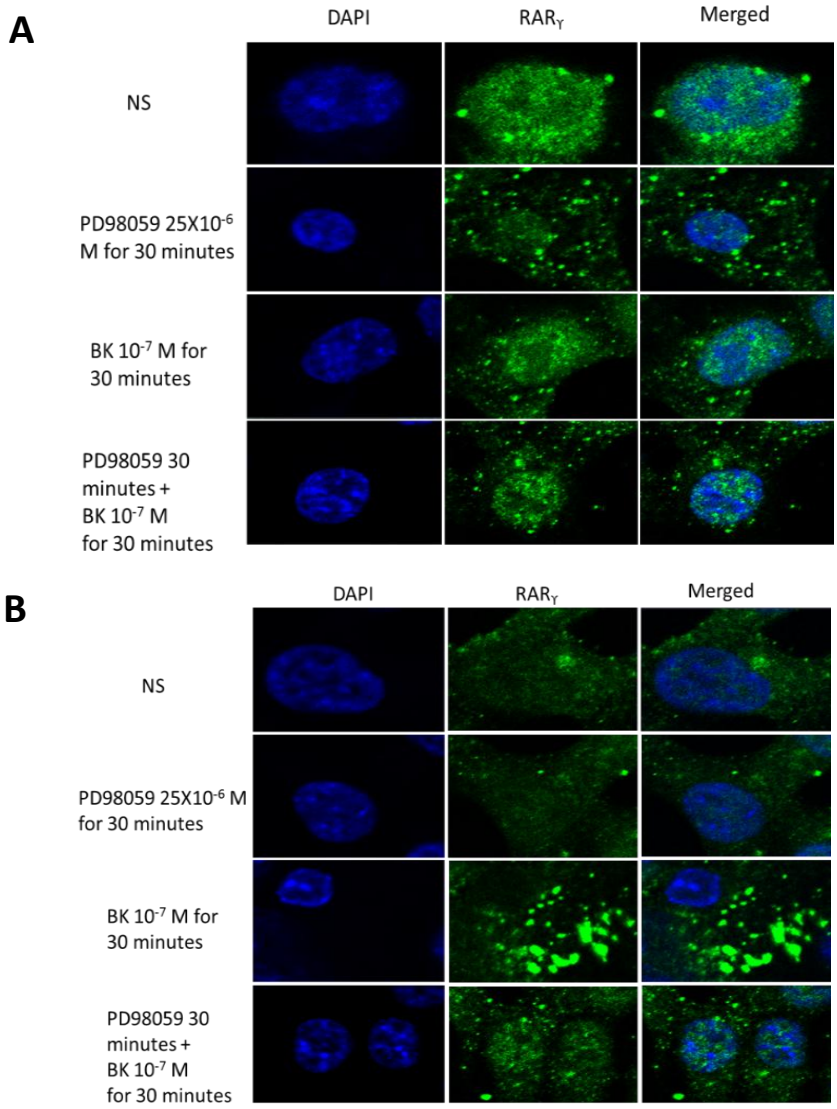

Figure 37. Regulation of intracellular localization of RARγ by serum starvation conditions in HCT116 p21-/- cells. (A) Starved for 30 minutes (B) Starved for 24 hours, and treated with bradykinin [10^{-7} M] or/and PD98059 [$25X10^{-6}$ M] for 30 minutes. Cells were immunostained using anti-rabbit RARγ antibody and nuclei were counterstained with DAPI. Cells were visualized at a magnification of 63x using confocal microscopy and are representative of several fields.

CHAPTER IV

DISCUSSION

Colorectal cancer has a high incidence and cancer-related mortality (Siegel et al., 2015). Although important progress has been achieved in the treatment strategies of colorectal cancer, the five-year survival rate is still relatively low of 65%. There is an urgent need for new and radical approaches for colorectal cancer therapy. So far, treatment strategies in the clinic have, to a certain extent, failed to make a significant achievement (Plumb & Halligan, 2015). Poor screening, follow-up, and chronic colorectal inflammation contribute to the poor prognosis of colorectal cancer.

The role of bradykinin and its receptors in inflammation has been well delineated (da Costa et al., 2014). Thus, their potential targeting in cancer therapy should be investigated since common colorectal cancer cases arise from a chronic inflammatory state involving multiple key players (Stewart et al., 2001). Moreover, retinoids are well-known for their cell differentiating and potent anti-proliferative and cell death effects and have been used in the cancer clinic (Ocker et al., 2003). Therefore, investigating the potential crosstalk between bradykinin and the retinoid receptors may ultimately reduce the inflammatory state for prevention and/or better treatment of colorectal cancer. First, we were interested in screening and characterizing the basal levels of bradykinin and several retinoid receptors and their modulation by bradykinin. We used a well- characterized *in vitro* human model of colorectal cancer of isogenic cell lines, namely HCT116, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$, with

different *p53* and *p21* status as these tumor suppressor genes are implicated in this cancer development.

To our knowledge, there are no previous reports that have examined the possible crosstalk between bradykinin and retinoid receptor signaling in cancer. Although retinoid receptors mostly exert their effects through nuclear receptors, however their non-genomic actions have been lately appreciated in signal transduction pathways (Schenk et al., 2014). Most importantly, the unique cytoplasmic subcellular localization of RARγ due to its N-terminal A/B domain, has been found to play a major role in coordinating many signaling cascades such as phosphorylation, RXR heterodimerization among others (Han et al., 2009). As a result, we were also interested in investigating whether bradykinin regulates the subcellular localization of retinoid receptors, in particular for RARγ.

We detected B1R, B2R, RARα, RARγ, and RXRα in the different colorectal cancer cell lines and did not observe any modulation of their basal transcript and protein levels by the tumor suppressor genes *p53* and *p21*. Moreover, the B1R agonist and bradykinin did not affect the cell growth of the different tested human colorectal cancer cell lines.

B1R agonist treatment did not regulate its own receptor protein levels in the tested colorectal cancer cells but induced B2R protein levels in HCT116 and HCT116 $p53^{-/-}$ cells which may be related to the *p21* status of the cells. Furthermore, bradykinin which is a high affinity B2R ligand increased its own receptor protein levels in HCT116 and HCT116 $p53^{-/-}$ cells but not in HCT116 $p21^{-/-}$ cells. This increase in B2R protein levels upon bradykinin treatment only correlated with increased transcript levels in HCT116 $p53^{-/-}$ cells.

We also investigated the potential crosstalk between B1R agonist and bradykinin regarding retinoid receptor expression in colorectal cancer cells. B1R agonist treatment significantly increased RARγ and RARα but did not affect RXRα protein levels in the tested human colorectal cancer cell lines. The induction in retinoid receptor proteins did not correlate with increased transcript levels and may be related to increased protein stability. On the other hand, bradykinin treatment only regulated RARγ gene expression where it increased transcript and protein levels were observed in HCT116 and HCT116 $p21^{-/-}$ treated cells but not in HCT116 p53-/- cells and may implicate *p53* status in the observed effect.

Since the protein kinase B, namely AKT, is a downstream target of bradykinin and has been found to be activated in several human cancers, targeting the AKT pathway for new drug discovery specifically in human colorectal cancer cells is central. Previous studies have shown that the AKT pathway gets hyperactivated in many types of human cancers (Altomare & Testa, 2005). However, our results showed the opposite effect, whereby AKT phosphorylation levels were rapidly decreased in bradykinin-treated colorectal cancer cells with intact *p21* status.

ERK1/2, a downstream target of bradykinin, was also investigated since this kinase is highly involved in cell proliferation, survival, and metastasis. ERK1/2 was found to be mutated and/or overexpressed in many types of cancer, and is therefore a main target in drug discovery whereby specific kinase inhibitors are being developed for the treatment of cancer. ERK1/2 activation was demonstrated to have anti-apoptotic activities, thus promoting cell survival (Roberts & Der, 2007). ERK1/2 status has not been characterized so far in these human colorectal cancer cell lines and our results show an induction of ERK1/2

phosphorylation by bradykinin in all tested cells, which constitutes an attractive target in colorectal cancer therapy.

We have interesting results regarding a possible spatiotemporal crosstalk between RARγ and B2R in HCT116 cells which varies upon bradykinin treatment duration. However, the subcellular localization of RARγ and B2R in HCT116 $p53^{-/-}$ cells does not seem to reflect a spatiotemporal crosstalk between these two receptors. Short-term treatment with bradykinin led to the nuclear retention of the RARγ possibly through pathways that do not involve direct B2R-RARγ crosstalk. However, in HCT116 $p21^{-/-}$ cells, short term treatment with bradykinin reflects a possible spatiotemporal crosstalk between both receptors within the cytoplasm, whereas long term treatment had no effect. Thus, the duration of treatment in HCT116 $p21^{-/-}$ cells is critical unlike the other two human colorectal cancer cell types. Interestingly, the B2R, known to be a G-protein coupled receptor usually found on the cell membrane of cells, could be located in the nucleus under certain conditions and in some cell types (Savard et al., 2008), was also found to be nuclear in HCT116 $p21^{-/-}$ cells. This reflects the presence of at least two types of B2Rs in these cells, one of which is nuclear and the other membranous, which might have distinct roles in mediating several cellular signals.

Bradykinin actions on short term and long term starved cells seem to be fully reverted by the ERK1/2 inhibitor, PD98059, which indicates a possible role of the ERK1/2 pathway in bradykinin actions. Our results indicate that ERK1/2 affects the subcellular localization and possibly the function of RARγ, depending on serum starvation conditions. It would be interesting to determine the effects of ATRA on bradykinin-induced subcellular localization of RARγ and whether these involve genomic or non-genomic effects.

Preliminary results indicate a differential regulation of the bradykinin and retinoid receptors by bradykinin in normal colorectal cells (data not shown) which requires further investigation in comparison to colorectal cancer cells. Interestingly, increased levels of B1R were observed in clinical samples of colorectal adenomas, which tend to evolve into colonic cancer (Zelawski et al., 2006). In addition, elevated levels of B2R were detected in hyperplastic polyps with no neoplastic potential (Zelawski et al., 2006). Understanding the complex mechanisms regarding the possible crosstalk between the bradykinin and retinoid receptors, with their major signaling pathways, could lead to novel targeted therapeutic strategies which may be beneficial to colorectal cancer patients.

So far, studies on the use of selective kinin antagonists or kinase inhibitors as potential therapeutic drugs have been limited (da Costa et al., 2014). Therefore, there is a need for further validation of kinin receptors as important therapeutic targets, alone and in combination with other drugs. Combination of bradykinin receptor antagonists and retinoids in colorectal cancer could constitute a potential cure for this type of cancer, but this should be preceded by a thorough evaluation of the use of bradykinin antagonists in some selected preclinical models and associated clinical indications as well as their mechanism of action.

It is crucial to elucidate how the actual intracellular levels and subcellular localization of retinoid receptors are controlled. Recent findings implicate extranuclear actions of retinoid receptors where RARγ cytoplasmic *versus* nuclear localization is regulated by complex interactions involving ATRA binding, and retinoid receptor phosphorylation and heterodimerization with RXRα (Han et al., 2009). Future studies will shed light on the effect of ATRA with or without B1R and B2R agonists on the subcellular localization of the

retinoid receptors and their downstream signaling pathway. RARγ subcellular localization and regulated signaling pathways may play a crucial role in the progression of colorectal cancer.

Despite the success of some anticancer drugs in clinical trials or in the clinic, the phenomenon of drug resistance is still a major drawback. Therefore, investigating alternative and novel approaches for overcoming drug resistance by targeting signaling pathways may lead to successful therapeutics. Specifically, a better understanding of the intracellular pathways initiated by kinins and retinoids, in addition to the possible crosstalk and their involvement in tumor progression, may be of great benefit as targets for the development of novel anti-cancer drugs.

A detailed proteomic approach to study bradykinin and retinoid signaling is worth investigating. Chromatin immunoprecipitation studies or microarray analysis will help in identifying genomic *versus* non-genomic approaches. Consequently, the synthesis of new pharmaceutical drugs with an extended therapeutic repertoire, reduced toxicity, and improved therapeutic index could be possible.

Re-evaluation, particularly *in vivo*, of the mechanism of action and functional roles of the B1R and B2R should be assessed, knowing that inflammation favors proliferation, angiogenesis, and ultimately tumor progression leading to poor patient prognosis. This should also be combined with a need for improved animal models such as chemically induced and transgenic, being closer to a humanized system.

Several limitations in this study need to be addressed including some experimental variability regarding the RT-qPCR results which displayed high variability. In addition, a

normal human colon epithelial cell line should be used to compare the alterations in bradykinin and retinoid signaling crosstalk in normal *versus* colorectal cancer cells

Importantly, although the tested colorectal cancer cell lines are resistant to ATRA (unpublished results), however the effect of this retinoid on retinoid signaling and crosstalk with bradykinin receptors should be deciphered in in these colorectal cancer cells. Testing ATRA in parallel with drugs that regulate bradykinin signaling pathway might combat drugs resistance and may work through genomic and non-genomic effects in these cells.

Furthermore a more in-depth investigation on other key players in both the retinoid and bradykinin signaling pathways which were not tackled during this research project, such as the inflammatory players NF-κB, IL-6 among others should be studied. Recently bradykinin was found to increase IL-6 levels *via* the B2R and ERK1/2 pathway and to regulate invasion and migration of colorectal tumor cells (G. Wang, Ye, Zhang, & Song, 2014). Furthermore, RARβ, should be tested for its tumor suppressor gene function and importance in the involvement and development of several tumors mainly colorectal cancer (Fang et al., 2010).

Finally, one of the limitations of these studies is that they are *in vitro* and not taking into consideration the tumor's microenvironmental alterations in response to treatment. By clarifying the molecular mechanisms underlying the tumor's microenvironment, more efficient therapeutic procedures modulating the microenvironment and helping in the treatment regimen could be, therefore, developed.

Despite advances in cancer therapeutics over the past few decades, relapse, treatment failure, and deaths from the majority of malignant diseases, namely cancer, remain

unsatisfactorily high (Meester et al., 2015). Our findings may lead to novel colorectal cancer therapies. Our results revealed interesting differential expression of the bradykinin and retinoid receptors in the three human colorectal cancer cell types, in response to various types of treatments. Thus, the very first characterization of these types of receptors, specifically in the human colorectal cancer cells, constitutes a panel from which further in-depth investigation should be run in order to decipher their role in colorectal cancer. The crosstalk between bradykinin and retinoid receptors in addition to their signaling pathways should be targeted, as to ultimately decrease the inflammatory state and get a more efficient effect of retinoids for the successful treatment of colorectal cancer.

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