A QUEST FOR THE UPSTREAM ACTIVATORS OF OCHRATOXIN A (OTA)-INDUCED MAPK/ERK1/2

by

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To my beloved family...

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ABSTRACT

A QUEST FOR THE UPSTREAM ACTIVATORS OF OCHRATOXIN A (OTA)-INDUCED MAPK/ERK1/2

Ochratoxin-A (OTA) is a mycotoxin, produced as a secondary metabolite by fungi belonging to *Aspergillus* and *Penicillium* genera. It has been recognized as a carcinogen in rodents and a possible carcinogen to humans. In our previous study, we demonstrated that OTA induces sustained MAPK/ERK1/2 activation in HK-2 cell line. Upstream activator of ERK1/2 protein activation remained obscure. In this study we screened a number of proteins via Western blot to determine if they cause ERK1/2 activation in OTA-exposed HK-2 cell line. We observed no dramatic increase in the expression levels of neither Integrin α and β subunits nor Integrin Linked Kinase (ILK). Also there was no discernable increase in the phosphorylation of Focal Adhesion Kinase (FAK) residues that might be involved in OTA mediated ERK1/2 activation under the experimental conditions employed in this study. Although a stable level of PKC phosphorylation was not observed, inhibition of various PKC isoforms reduced OTA-induced ERK1/2 activation. These results suggest PKC as a possible upstream activator in the activation of MAPK/ERK1/2 in OTA-treated HK-2 cells. Moreover, modulation of G protein-coupled receptor (GPCR)-dependent signaling through pertussis and cholera toxins suppressed ERK1/2 activation in response to OTA treatment. In addition, chemical agents that increase intracellular cAMP levels subdued OTA-induced ERK1/2 activation considerably. We also observed that H-89, a cAMP-dependent kinase (PKA) inhibitor, augments OTA-induced activation of ERK1/2. Taken all together, this study suggests that OTA may cause stimulation of a putative GPCR that results in a decrease in intracellular cAMP levels and ultimately activation of MAPK/ERK1/2 in HK-2 cell line where PKC may be an intermediary in relaying signal from GPCR to ERK1/2.

ÖZET

OKRATOKSİN A (OTA)-TEŞVİKLİ MAPK/ERK1/2'NİN ÜST YOLAK AKTİVATÖRÜNÜN BULUNMASI İÇİN BİR ARAŞTIRMA

Ochratoxin-A (OTA) *Aspergillus* ve *Penicillium* cinsinden mantarların ikincil metaboliti olarak üretilen bir mikotoksindir. Kemirgenlerde kanser yapıcı etkisi gözlenmiş olup, insanlarda da kanser yapması olasıdır. Önceki çalışmamızda OTA'nın HK-2 hücre hattında sürekli MAPK/ERK1/2 aktivasyonunu teşvik ettiğini göstermiştik. ERK1/2 protein aktivasyonunun üst yolak belirleyeni ise karanlıkta kalmıştı. Bu çalışmada ERK1/2 aktivasyonunda rol alıp almadıklarını belirlemek amacıyla birtakım proteinleri Western blot yöntemi ile inceledik. Integrin α ve β alt birimlerinin veya Integrin Bağlantılı Kinaz (ILK) ifade seviyelerinde anlamlı bir artış gözlemedik. Ayrıca OTA teşvikli ERK1/2 aktivasyonunda rol oynayabilecek Fokal Adezyon Kinaz (FAK) proteininin fosforilasyonunda ayırt edici bir artış da gözlenememiştir. Kararlı düzeyde bir PKC fosforilasyonu gözlenmese de, bazı PKC izoformlarının kimyasal inhibitörlerle baskılanmasının OTA teşvikli ERK1/2 aktivasyonunu azalttığı belirlenmiştir. Bu sonuçlar PKC'nin, OTA verilmiş HK-2 hücresinde MAPK/ERK1/2 aktivasyonunun bir üst yolak etkeni olabileceğini önermektedir. Dahası, boğmaca ve kolera toksinleri ile G protein-kenetli reseptör (GPCR) bağımlı sinyal iletim yolağının modülasyonu, OTA teşvikli ERK1/2 aktivasyonunu baskılamıştır. Ek olarak hücre içi cAMP düzeyini arttıran kimyasal ajanlar OTA-teşvikli ERK1/2 aktivasyonunu önemli ölçüde düşürmüştür. Bir cAMP bağıntılı kinaz (PKA) inhibitörü olan H-89'un ise ERK1/2 üzerindeki OTA teşviğini arttırdığını gözledik. Hepsi bir arada, bu çalışma OTA'nın HK-2 hücrelerinde olası bir GPCR'yi tetiklemesiyle hücre içi cAMP seviyesinde bir düşüşe, onun sonucunda da MAPK/ERK1/2 aktivasyonuna neden olabileceğini ve GPCR'den ERK1/2'ye sinyalin iletilmesinde PKC'nin aracı olabileceğini önermektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

°C	Degree Celsius
kDa	Kilodalton
RLU	Relative Light Units
rpm	Revolutions per minute
V	Volt
μg	Microgram
μl	Microliter
μM	Micromolar
g	Gram
L	Liter
mg	Milligram
min	Minute
ml	Mililiter
mM	Milimolar
ng	Nanogram
nM	Nanomolar
α	alpha
β	beta
δ	delta
γ	gamma
λ	lambda
μ	mu
θ	theta
ζ	zeta

LIST OF ACRONYMS/ABBREVIATIONS

COS1	Monkey Fibroblast Cells
DU145	Human Prostate cancer Cell Line
HEK293	Human Embryonic Kidney Cells
HK-2	Human Kidney Proximal Tubular-2 Cell Line
LNCaP	Human Prostate Adenocarcinoma Cells
NIH3T3	Mouse Fibroblast Cell Line
PC12	Rat Pheochromocytoma cCell Line
PC3	Human Prostate cancer Cell Line
Swiss3T3	Mouse Embryonic Fibroblast Cells
FDA	Food and Drug Administration
IARC	The International Agency for Research on Cancer
ATCC	American Type Culture Collection
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleicacid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtOH	Ethanol
FBS	Fetal Bovine Serum
NaCl	Sodium Chloride
OTA	Ochratoxin A
RNA	Ribonucleicacid
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
TBS	Tris-buffered saline
TBS-T	Tris Buffered Saline-Tween
tRNA	Transfer RNA

cAMP	3',5'-cyclic adenosine monophosphate
ERK1/2	Extracellular Signal-Regulated Kinases 1/2
FAK	Focal Adhesion Kinase
GPCR	G-Protein Coupled Receptor
ILK	Integrin-Linked Kinase
KIM-1	Kidney Injury Molecule-1
MAPK	Mitogen-Activated Protein Kinase
PKC	Protein Kinase C
6-OHDA	6-hydroxydopamine
8-bromo	8-Bromoadenosine $3', 5'$-cyclic monophosphate
CTX	Cholera Toxin
H-89	N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride
IBMX	3-isobutyl-1-methylxanthine
ISO	isoproterenol hydrochloride
PMA	Phorbol 12-myristate 13-acetate
PTX	Pertussis Toxin
STS	Staurosporine
CO_2	Carbon dioxide
dH_2O	Distilled water

1. INTRODUCTION

1.1. Ochratoxin-A (OTA)

Ochratoxin-A (OTA) is one of the most abundant mycotoxins affecting animal and human health [1]. It is produced by fungi grown on improperly stored or processed food and feed [2]. OTA was first identified in 1965 as a toxic metabolite produced by a widespread fungi species *Aspergillus ochraceus* [3]. It is now known that *Penicillium* and *Aspergillus* genera of fungi produce OTA as a secondary metabolite [4]. These fungi produce OTA in various climate conditions and consequently contaminate food commodities [5]. Its chemical structure is presented in Figure 1.1.

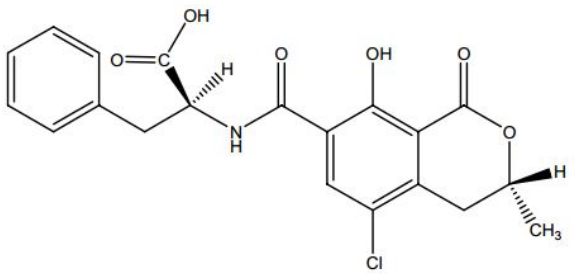

Figure 1.1. Chemical structure of Ochratoxin-A [6].

OTA can be found as a natural contaminant in a wide range of foods such as cereals and derivatives, wine, grape, chocolate, coffee, cocoa, spice, dried fruits, etc [7]. Additionally, contamination of animal feed with OTA gives rise to ruins of OTA found in blood serum organs such as kidney and liver [8]. Due to being a worldwide contaminant for food and feed, human population is frequently exposed to OTA in daily life.

OTA is absorbed in the small intestine and gastrointestinal system, and finally transported to the kidneys via the circulatory system. Although OTA is excreted from the kidney tubules by organic anion transport proteins at first, it is reabsorbed through proximal tubular cells in nephron segments. This reabsorption causes accumulation of OTA in the proximal tubular cells. Therefore, kidney has been viewed as the main target organ for OTA toxicity [9]. As an extremely stable compound, OTA can bind

to plasma proteins, especially serum albumin, thus its half-life exceeds two weeks in human blood circulation [10, 11].

1.1.1. Toxic effects of OTA

There are several *in vivo* studies performed in a range of animal species that show pathological effects of OTA on kidney cells. In F344 male rats fed with OTA for 13 weeks, the kidney was damaged seriously in many aspects. There was an increase in the cytoplasmic vacuole formation in epithelial cells. Additionally, in renal tubule epithelial cell membranes, kidney injury molecule-1 (Kim-1) which is a kidney damage specific biomarker was discovered. Furthermore, indicators of proliferation and carcinogenicity were detected in kidney cells. [12]. Another study on pigs has revealed that OTA contaminated feed consumption causes higher ratio of nephropathy [13–16].

In certain Balkan regions OTA is a common contaminant of food, and Balkan Endemic Nephropathy (BEN) is a prevalent disease among people living in that region [17]. OTA is now reputed as a causative agent for BEN and associated Urinary Tract Tumors (UTT) since the people having this disease show high blood concentrations of OTA compared to the healthy individuals. These evidence indicate not only the nephropathic feature of OTA, but also its carcinogenic property manifested in the kidneys [18].

OTA is also considered to be hepatotoxic based on the evidence that OTA triggers oxidative damage in rat liver after oral dosage [10]. Besides its nephrotoxic and hepatotoxic feature, OTA has also been deemed as neurotoxic [19], and immunotoxic [20, 21] to organisms. Considering all the data provided in the literature, OTA was accepted as a possible carcinogen to humans in 1993 by International Agency for Research on Cancer (IARC) [22].

1.1.2. Mechanism of Action of OTA

OTA has been one of the most important mycotoxins in cancer and toxicology fields, and there are several *in vivo* and *in vitro* studies performed in order to show carcinogenic and nephropathic effects of OTA. However, the mechanism of action for OTA remains elusive. There are controversial claims that have been proposed about the mode of action of OTA. These claims can be classified as genotoxic and non-genotoxic mechanisms.

1.1.3. Genotoxic effects of OTA

Some studies argue that OTA shows its toxic effect directly on DNA. OTA is claimed as a genotoxic chemical substance binding to DNA and promoting DNA adduct formation which increases mutation rates and consequently tumor formation. *In vivo* experiments performed on mice and rats demonstrated that OTA-DNA adducts were formed in kidneys and liver with only a single dose of OTA [23, 24].

1.1.4. Non-genotoxic effects of OTA

Investigations claiming an opposing view argue that OTA is a non-genotoxic substance that induces toxic effect by epigenetic mechanisms [25]. Oxidative stress is one of the most important cellular disturbance which is caused by OTA exposure resulting in damage to DNA and proteins [26]. Some observations claim that OTA causes ATP depletion which leads to inhibition of mitochondrial oxidative phosphorylation [27]. OTA is a phenylalanine-dihydrocumarin derivative and because of its resemblance to phenylalanine, OTA is claimed to reduce protein synthesis by binding to phenylalanine tRNA-synthetase [28, 29]. OTA has also been claimed for giving rise to enhanced lipid peroxidation [30].

OTA is also known to increase apoptotic signaling mechanisms in the cell. *In vivo* and *in vitro* studies show that OTA can cause an increase in the expression of genes related with apoptotic pathway [31, 32]. In an experiment on rats, OTA was

demonstrated to increase the percentage of cells entering apoptosis [33].

Proposed non-genotoxic mechanisms of OTA also include interference with various signal transduction pathways depending on cell type and dosage [26, 32, 34–37].

1.2. Mitogen-Activated Protein Kinase/Extracellular Signal-regulated Kinases 1/2 (MAPK/ERK1/2)

There are multiple MAPK pathways in eukaryotes which are responsible for regulation of a variety of cellular activities from gene expression, cell proliferation, differentiation to cell survival and apoptosis. In mammals, four distinct groups of MAPKs have been characterized so far: extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), c-Jun amino-terminal kinases (JNK1, JNK2, JNK3), p38 mitogen-activated protein kinases (with α, β, γ, δ isoforms), and ERK 5 [38]. Each family of MAPKs is composed of a set of three sequentially acting evolutionarily conserved kinases : a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). MAPKKKs are serine/threonine kinases activated through phosphorylation as a result of their interaction with a small GTP-binding protein of Ras/Rho family in response to extracellular stimuli. Activation of MAPKKK results in the activation of MAPKK via phosphorylation, which leads to stimulation of MAPK activity through phosphorylation on threonine and tyrosine residues. Activation of MAPK leads to phosphorylation of substrates on serine and threonine residues [39]. General cascade of MAPK family is illustrated in Figure 1.2. ERK1/2, p38 and JNKs kinases are the most extensively studied kinases in vertebrates [40].

1.2.1. Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2)

ERK1/2 signaling has been known to play a central role in the induction of cellular processes such as differentiation, proliferation, cell survival, migration and apoptosis [41]. In the general signaling cascade, ERK1 and ERK2 are activated via phosphorylation by MEK1 and MEK2. Ras-mediated phosphorylation of Raf activates MEKs (MAP2Ks) through phosphorylation. Due to this cascade MAPK/ERK signaling path-

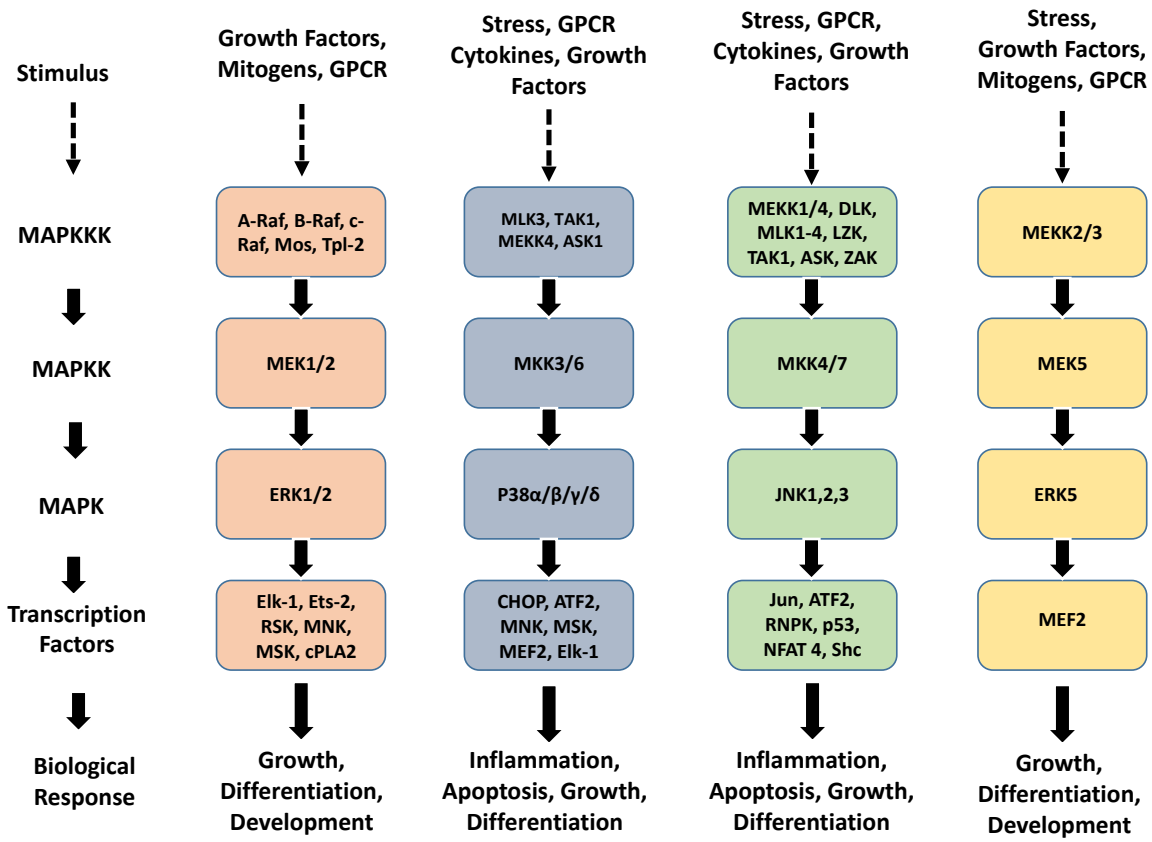

Figure 1.2. Schematic representation of MAPK signaling pathways (Illustration reproduced courtesy of Cell Signaling Technology, Inc.).

way has been considered as Ras-Raf-MEK-ERK pathway [42, 43].

Even though ERK1/2 activation has generally been associated with cell survival and proliferation, a number of studies demonstrate that activation of ERK1/2 can also mediate cell death depending on the stimuli and cell types which are involved [44, 45]. Chemical inhibition of ERK1/2 was demonstrated to cause attenuation in cisplatin-induced apoptosis in mice kidney [46]. In addition to this, an *in vitro* study showed that ERK promotes cell death in cerebellar granule neurons (CGN) [47].

Our previous results demonstrated that OTA inhibits cell growth by giving rise to cytotoxic and apoptotic cell death in HK-2 proximal tubular cell lines. It was also determined that OTA causes sustained activation of MAPK/ERK1/2 and PI3K/Akt signaling pathways in HK-2 cells. Besides, chemically or genetically inhibition of MEK/ERK1/2 signaling was shown to reduce OTA-induced ERK activation and subse-

quently apoptotic and necrotic cell death. This shows that activation of MEK/ERK1/2 signaling inflicts cell death seen in OTA-exposed HK-2 cells [32].

Although it is known that OTA causes apoptotic cell death in HK-2 cells through ERK activation, the molecular mechanisms that define OTA-induced ERK1/2 activation remains poorly understood.

1.3. Integrin-Linked Kinase (ILK)

Integrins are a large family of transmembrane proteins which act as a mediator for cell-cell and cell-extracellular matrix (ECM) interaction [48]. These evolutionary old cell adhesion receptors have regulatory roles in controlling cell migration, proliferation, survival and differentiation [49]. Integrins are found as heterodimers which are composed of α- and β- subunits on the cell surface. They differ from each other with regards to ligand-binding properties or subunit composition [50]. The α subunit of integrin is comprised of a seven-bladed β-propeller repeats. This repeat domain is connected to thigh, calf-1 and calf-2 domain. Together, they form the leg structure and serve to support the integrin head. The β subunit includes a plexin-sempahorin-integrin (PSI) domain, a hybrid domain, and four cysteine-rich epidermal growth factor (EGF) repeats (Figure 1.3). In heterodimer formation, α subunit determines integrin ligand specificity and has the feature of connecting to cytoskeleton and affecting multiple signaling pathways [50, 51].

Integrins become activated through conformational changes as a consequence of ligand binding and they cluster on the plasma membrane. Integrins are able to achieve signaling by interacting with proteins that have a bridging role between integrins and downstream components. One of these proteins is the Integrin-Linked Kinase (ILK) that interacts with integrins on the cytosolic domain of β subunit of integrin heterodimer. [49, 52].

ILK is a 59 kDa serine/threonine protein kinase having crucial roles in cell proliferation, survival, cell-adhesion and migration [53]. ILK is comprised of three distinct

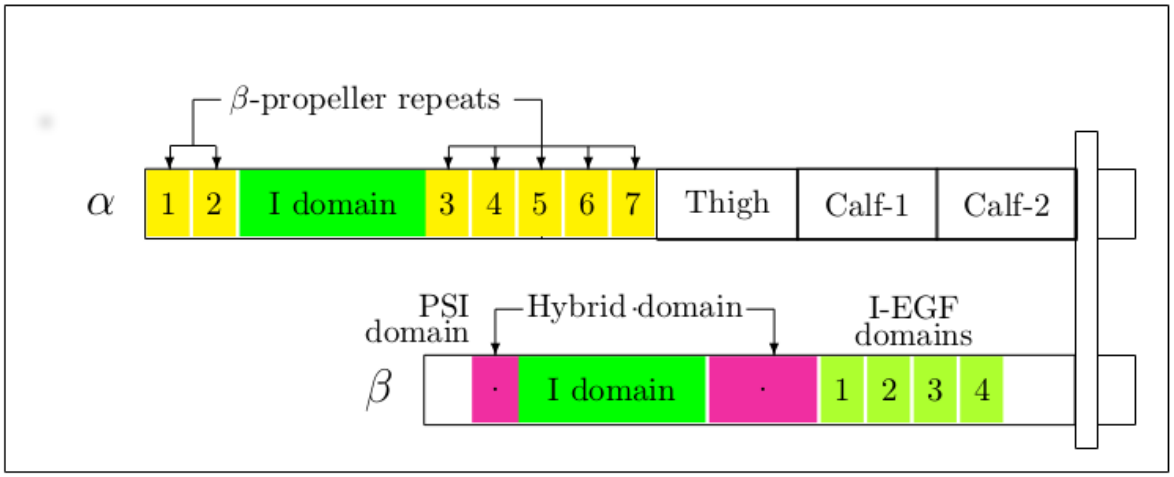

Figure 1.3. Representation of alpha and beta subunit of an integrin heterodimer (Adapted and modified from Barczyk *et al.*, 2010).

regions structurally: i) Four ankyrin repeats and İİ) a pleckstrin homology (PH)-like domain (PIP3 binding) at the amino (NH2-) terminus, iii) a kinase-like domain at the carboxy (COOH-) terminus [54]. The structure of ILK is illustrated in Figure 1.4.

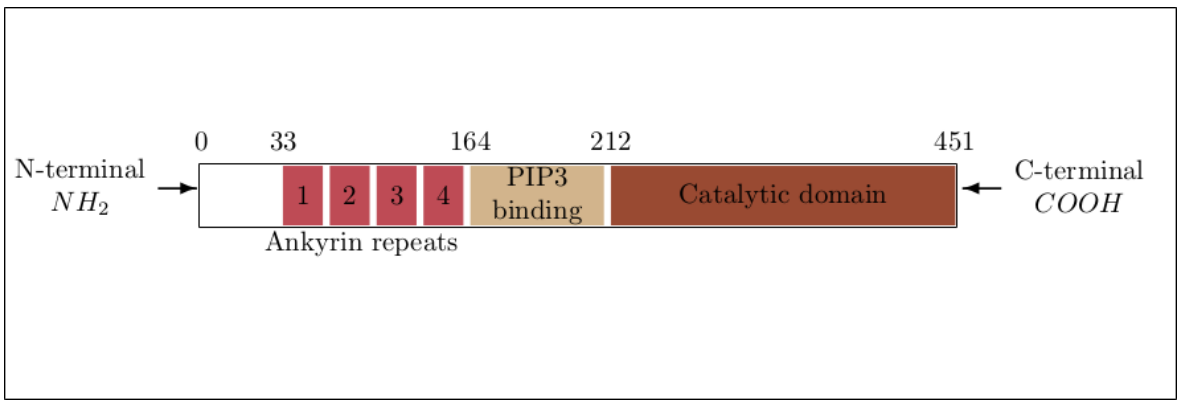

Figure 1.4. Structural domains of ILK (Adapted and modified from Hannigan *et al*, 2005).

There are several studies showing the relation between ILK activation and increased activity in downstream signaling pathways such as MAPK/ERK1/2 pathway. In human cardiomyocytes ILK-overexpression results in the activation of ERK1/2 [55]. In addition, in ILK transgenic mice, ILK overexpression causes increased activation of ERK1/2 [53]. ILK also is deemed as a possible activator of ERK1/2 in muscle differentiation [56].

In an unpublished work from our laboratory, it was determined that the expression of a cytoskeletal protein, actinin-4, has been increased under 24 hours of 10μOTA exposure in HK-2 cells. Proteomics approach was utilized to determine global changes in the expression of proteins under the OTA exposure in HK-2 cell line (Figure 1.5). Actinin-4 is known to be a member of integrin signaling and a participant of ILK-mediated linkage of integrins to the actin cytoskeleton in cells [57]. The previous data made us think that ILK might act as a scaffold protein for integrin and cytoskeleton interaction under OTA-induced ERK1/2 activity in HK-2 cells.

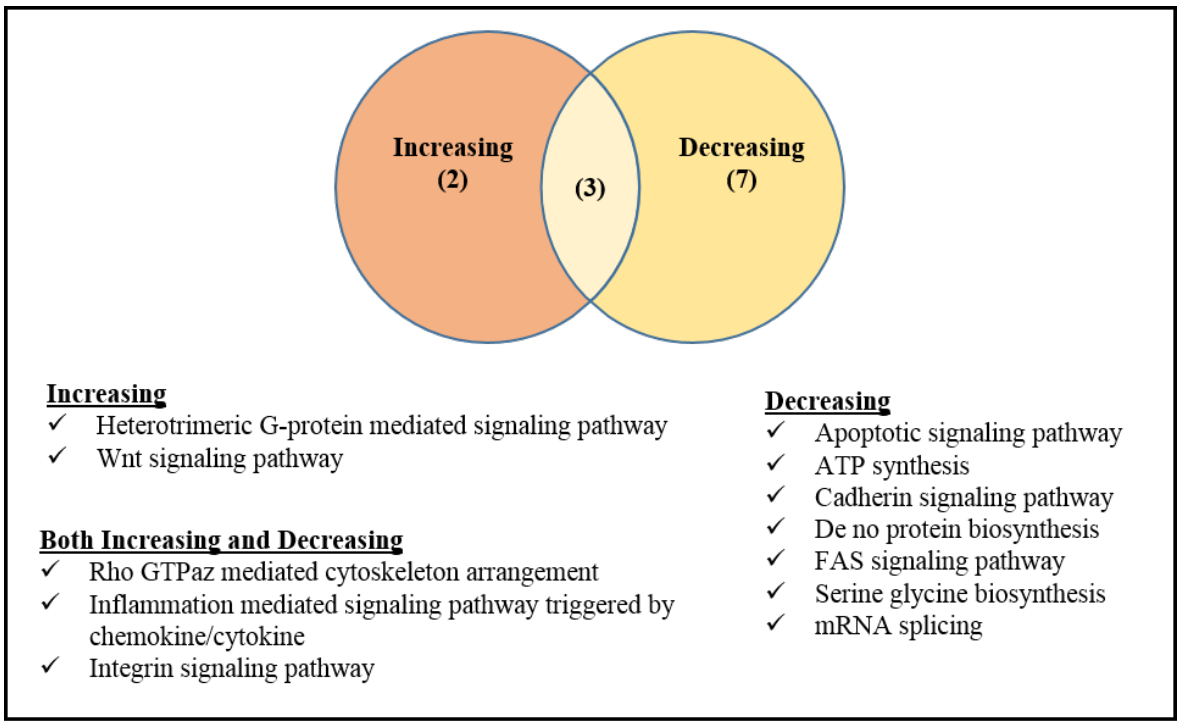

Figure 1.5. Determination of the proteins responding in terms of expression change under OTA exposure. Signaling pathways in which proteins of changing expression are involved were found by using PANTHER database.

1.4. Focal Adhesion Kinase (FAK)

Focal Adhesion Kinase (FAK) is a member of protein tyrosine kinase family and has been known to have a prominent role in cellular processes such as cell cycle progression, growth factor signaling, and cell migration [58]. When we look at the structure of FAK, it is clearly seen that FAK is composed of a central catalytic domain accompanied by two large non-catalytic N-terminal and C- terminal domains [59]. The N-terminal domain is called FERM (band $f$our point one, $e$zrin, emphradixin,

and emphmoesin) domain. Even though the role of FERM domain in FAK protein is unclear, there are *in vitro* studies demonstrating that N-terminal domain binds to cytoplasmic region of β-integrin subunit [60]. It is also claimed that N-terminal domain of FAK mediates the interaction with integrins and growth factors receptors [61]. The C-terminal domain of FAK is known to be rich in protein-protein interaction sites. There is a sequence in this domain called focal adhesion targeting, FAT, that is responsible for targeting FAK to adhesion complexes [62]. The structure of FAK is shown in Figure 1.6.

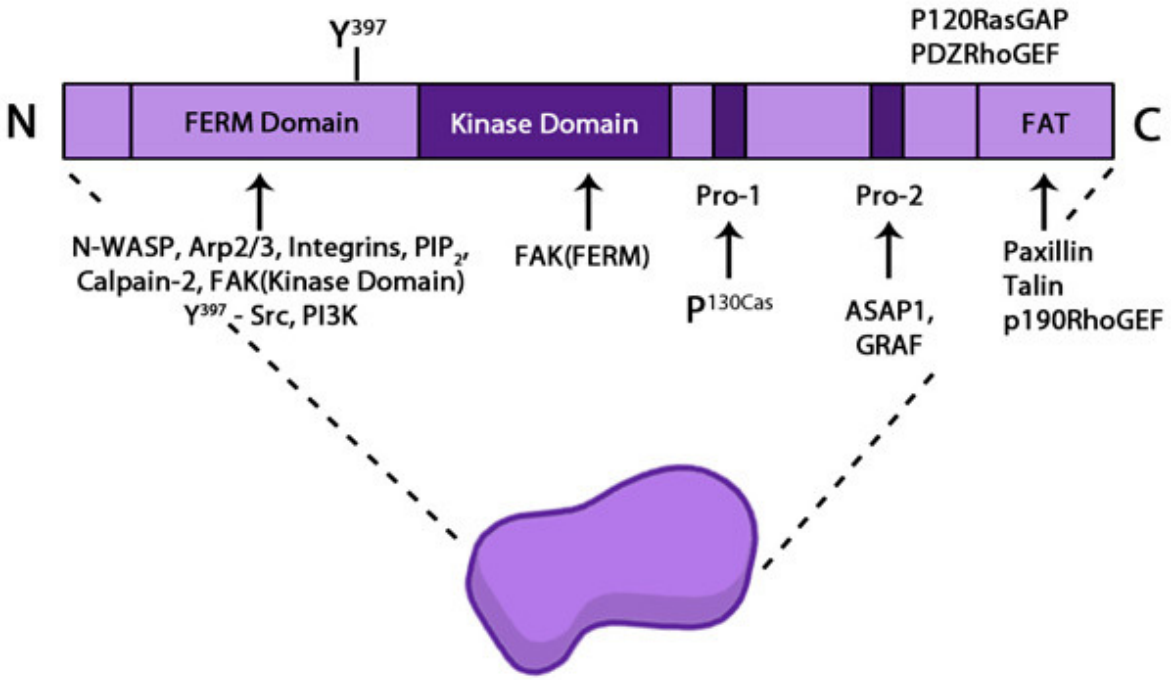

Figure 1.6. Structural domains of FAK (Used by permission from Mechanobiology Institute, National University of Singapore).

Upon integrin engagement on the plasma membrane FAK phosphorylation occurs and this phosphorylation results in the phosphotyrosine docking site formation for several classes of signaling molecules [63]. Tyr 397 residue is the autophoshorylation site of FAK protein and the phosphorylation on Tyr397 creates a high affinity binding site for the SH2 domain of Src family kinases, phosphoinositide 3-kinase (PI 3-kinase), phospholipase C (PLC)-γ and the adapter protein Grb2. Binding of these proteins leads to the formation of the kinase complex [64]. Tyr397-dependent activation of FAK continues with additional phosphorylation on Tyr576/577 and Tyr925 residues

which are in the catalytic domain and carboxy-terminal region, respectively [65].

MAPK/ERK1/2 pathway has shown to be one of the downstream signaling events that is regulated by FAK. Osteogenesis is induced via activation of the MAPK/ERK1/2 pathway and FAK is an upstream effector of this activation in human mesenchymal stem cells [66]. FAK-dependent activation of ERK1 underlies Toll-Like Receptor (TLR)-mediated stimulation of eosinophils [67]. There are also other studies claiming that mycotoxin contamination including OTA, may result in regulation of FAK in various cell types [68, 69]. Considering the FAK binding to integrin β subunits and subsequently causing many biological responses in the cell, FAK might be an upstream regulator for the sustained activation of ERK1/2 in OTA-exposed HK-2 cell line.

1.5. Protein Kinase C (PKC)

Protein Kinase C (PKC) is a large family of serine/threonine protein kinases which have key roles in certain cellular physiological processes such as cell proliferation, cell death, cell differentiation and transformation [70]. PKC enzymes have different isozymes and they comprise three different classes; "conventional", "novel" and "atypical" isozymes [71]. All members of PKC family have a common structure. They are single polypeptides composed of an N-terminal regulatory region and a C-terminal catalytic region which is demonstrated in Figure 1.7 [70].

The region depicted in orange color is the catalytic domain included in the C-terminus of the protein. There is a high homology among PKC isoforms in the catalytic domain. Threonine residue in the activation loop is phosphorylated for catalytic activity and generally it is an important first step for maturation of the newly synthesized protein [72]. All three classes of PKC have a turn motif and a C-terminal hydrophobic motif that are depicted as yellow circles in the Figure 1.7. The brown circle on the C-terminus of atypical PKC enzymes refers to a glutamic acid residue instead of a hydrophobic motif. The regulatory domains shown in blue and red are located in the N-terminal part of the enzymes. [70, 71].

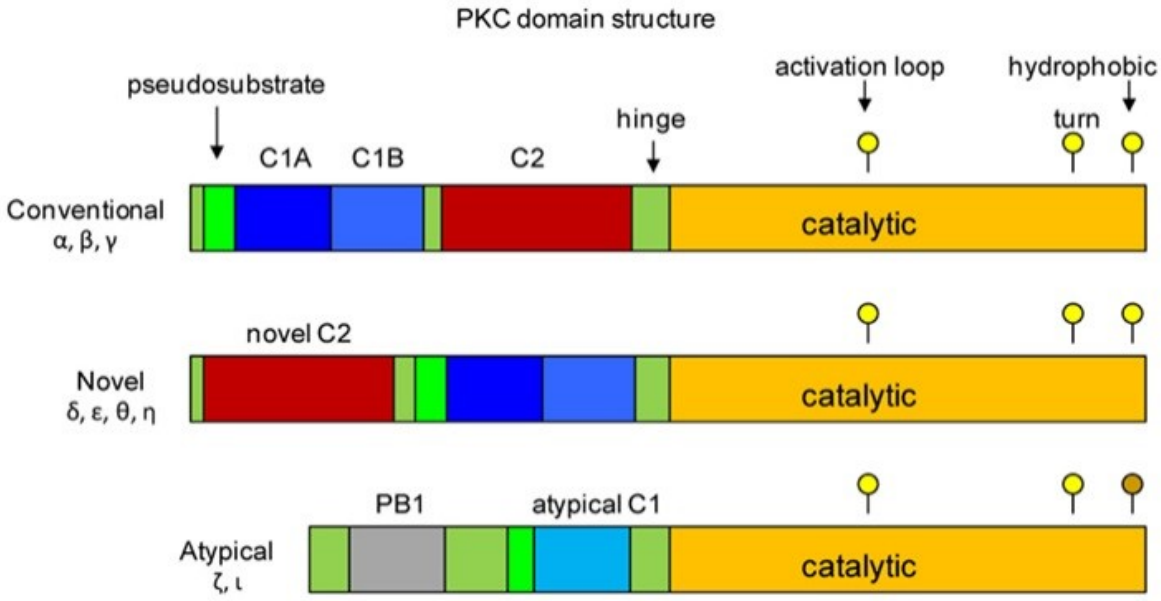

Figure 1.7. Illustration of PKC domains (Adapted from Gibbs *et al*, 2012).

The three groups of PKC enzymes differ from each other based on not only some differences in their structure, but also their activators. Conventional isoforms are comprised of PKCα, PKCβI, PKCβII, PKCγ and do require both calcium (Ca^{2+}) and diacylglycerol (DAG) for their activation. Novel isoforms are PKCδ, PKCϵ, PKCη, and PKCθ. The activity of these isoforms occurs independently from calcium ions. They can only be activated by DAG. Atypical isoforms refer to PKCζ, PKCι, and PKCλ and they do not require neither calcium nor DAG for activation. They are known to be dependent on different lipid metabolite messengers [72–74]. In the activation of conventional PKC enzymes, an external stimulus binds to its specific plasma membrane receptor resulting in the activation of Phospholipase C (PLC) and hydrolysis of phosphatidylinositol in the cell. The activated PLC hydrolyzes inositol triphosphate 2 (PIP2) into DAG and inositol trisphosphate 3 (IP3). Then IP3 opens intracellular calcium channels and calcium is released from intracellular stores resulting in an increase in the concentration of cytosolic calcium ions. Calcium binds to C2 domain and DAG binds to C1 domain of conventional PKC and enzyme gets activated. Finally, PKC activation leads to regulation of many proteins as an upstream effector [70, 75]. The mechanism of activation of PKC enzymes is illustrated in Figure 1.8.

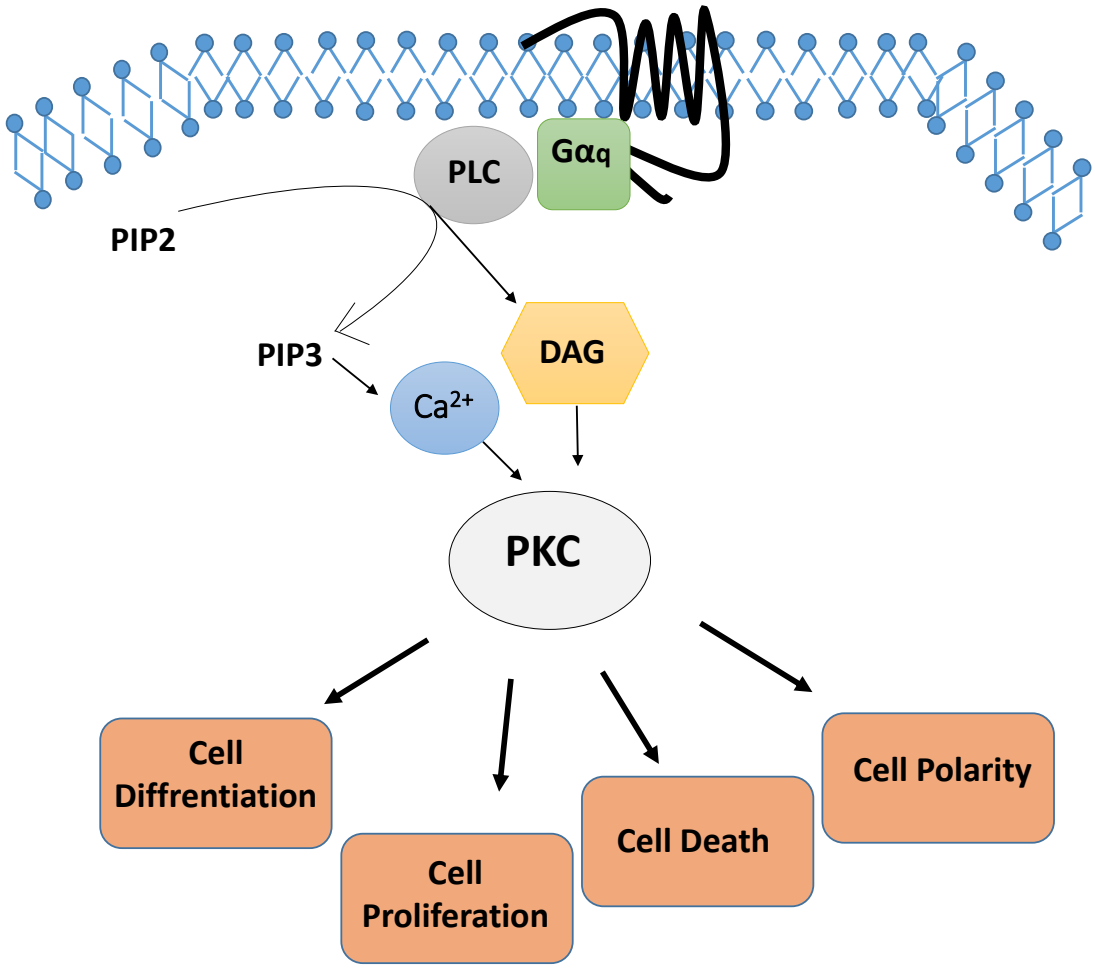

Figure 1.8. Activation of PKC by upstream effectors (Adapted and modified from Palaniyandi *et. al.*, 2009).

Following the identification of these different groups of isozymes, there has been a burgeoning of investigations on PKC. There are several studies indicating that PKC enzymes have a role in MAPK/ERK1/2 signaling pathway. In COS1 and NIH3T3 fibroblast cell lines, it has been observed that MAPK/ERK1/2 can be activated by PKCδ enzyme [76]. In addition, it is already known that PKCζ has an activating effect on MAPK/ERK1/2 pathway in human alveolar macrophage cells [77]. Furthermore, ERK1/2 has been shown to be activated by PKCα and PKCϵ in human glioma cells [78]. In a study which was performed with kidney rat, OTA was observed to increase the phosphorylation of PKC enzyme. In addition to this, increased PKC activation was correlated with a downstream activation of MAPK/ERK1/2 pathway [79]. Reactive Oxygen species (ROS) production was shown previously to be depending on OTA exposure in some *in vitro* studies [80, 81]. The induction of ROS production in cells

was also found to be related with the activation of PKC and ERK1/2 signaling [82]. Considering the studies so far, it might be plausible to think that PKC might have a regulatory effect on the sustained activation of MAPK/ERK1/2 pathway in OTA exposed HK-2 cell line.

1.6. G Protein-Coupled Receptors (GPCRs)

G protein-coupled receptors (GPCR), also known as seven transmembrane domain receptors (7TM) are plasma membrane receptors recognizing extracellular signals and ultimately triggering the signal transduction pathways in the cell [83]. GPCRs are known to be the largest receptor family in the genome by far. Since there are more than 800 ($\sim$ 950) GPCRs in the human genome, this protein family has become the biggest field of biological investigation [84]. Odors, pheromones, light sensitive compounds, hormones, neurotransmitters and many xenobiotics are the ligands which activate the GPCRs. Therefore, GPCRs are very popular in pharmaceutical industry because of the fact that GPCRs are the target of most of the drugs [85]. As illustrated in Figure 1.9 GPCRs have seven alpha helices in the membrane and an amino terminal in the extracellular part and a carboxyl terminus in the intracellular portion [86].

1.6.1. G Proteins and GPCR Signaling Activation

After binding of a ligand to GPCR, some conformational changes occur in the receptor structure and the receptor gets activated [87]. Activated receptor recognizes and couples to Guanine-nucleotide binding proteins or simply known as heterotrimeric G proteins. Heterotrimeric G proteins are composed of three protein subunits which are called; G alpha (G_α), G beta (G_β), and G gamma (G_γ) [83, 88]. Alpha and gamma subunits have lipid tails which help maintenance of G proteins being anchored to the membrane [89]. When there is no ligand bound to the receptor, Gα subunit is in inactive state as it is bound to Guanosine diphosphate (GDP). In case of GPCR activation, GDP dissociates from the complex[90]. With the dissociation of GDP, guanosine triphosphate (GTP) found freely and abundantly in the cytosol can now replace GDP. Binding of GTP in place of GDP causes a further conformational change

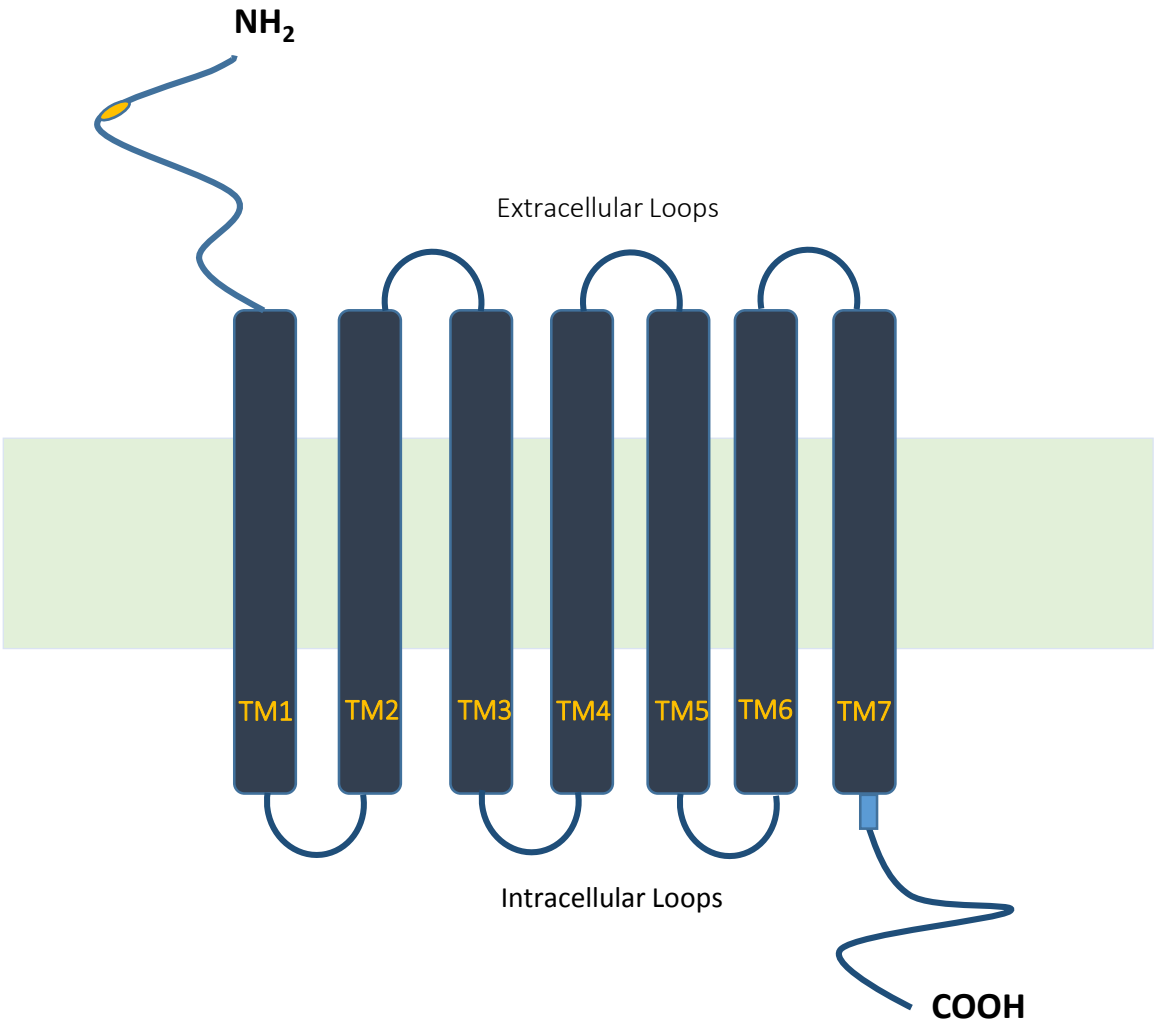

Figure 1.9. Physical structure common to all GPCRs (Adapted and modified from Kobilka, 2007).

in the G protein, and finally G protein now become active with its three subunits. In general, activated Gα subunit dissociates from the activated complex and Gβ-Gγ components activate the other target proteins. The activated target proteins relay the signals to the other components in the pathway. After the transduction is completed, GTP dissociates from the Gα subunit to be replaced by GDP, and the trimeric G protein complex turns back into its inactivate state [89, 91] (Figure 1.10).

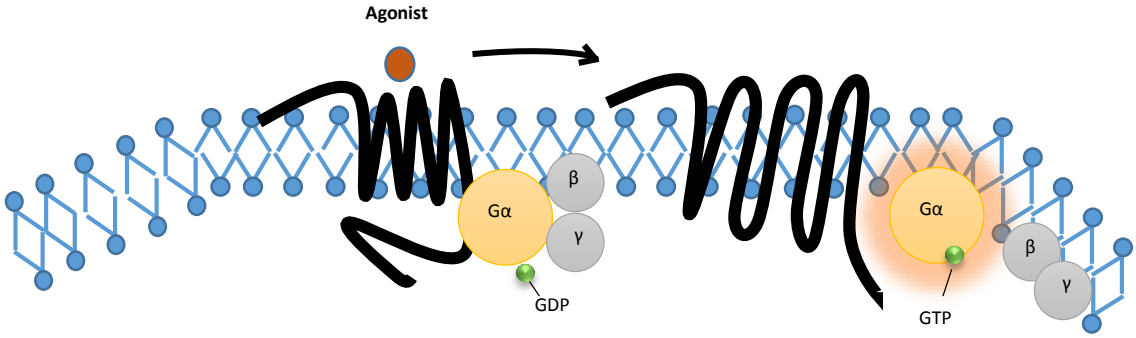

Figure 1.10. Activation of GPCR and nearby G proteins (Adapted and modified from Li, J. *et al.*, 2002).

There are other ways for the termination of GPCR signaling which is called GPCR desensitization [92]. Right after ligand binding to its own receptor and causing conformational change, GPCR-regulating kinases (GRKs) are recruited. GRKs phosphorylate receptor at C-terminal tail on serine/threonine residue. Following the phosphorylation, receptor becomes a substrate for binding of β-arrestin proteins. β-arrestin uncouples receptor and G-protein so that the agonist-induced response is desensitized. In addition to hindering G-protein, β-arrestin binding also causes internalization of the receptor via clathrin-mediated endocytosis. Other second-messenger dependent kinases can also phosphorylate GPCR and induce desensitization process [92, 93]. Due to being the largest class of receptors, GPCRs can give rise to activation of several effectors such as adenylate cyclase and phospholipase after stimulation. These effectors have the ability to influence intracellular concentrations of seconder messengers like cyclic AMP, diacylglycerol, inositol 1,4,5 triphosphate and Ca^{2+} and as a consequence stimulate the activity of many targeted proteins such as protein kinase A and protein kinase C and their downstream members. It is now quite clear that many 7TM receptors can interfere with the Ras-Raf-MEK-ERK pathway (Figure 1.11) [94–97]. It was previously shown also that mycotoxin exposure can result in deregulation of ERK1/2 signaling through GPCR activation in neuroepithelial cells [98]. In our proteomics data, some proteins which are related with G protein-dependent signaling were observed to increase under OTA exposure in HK-2 cells (Figure 1.5). Again, considering data available in the literature relating the GPCR and ERK1/2 activation and the proteomics study conducted in our laboratory, 7TM receptors are our possible candidates to play a role in OTA-induced activation of MAPK/ERK1/2.

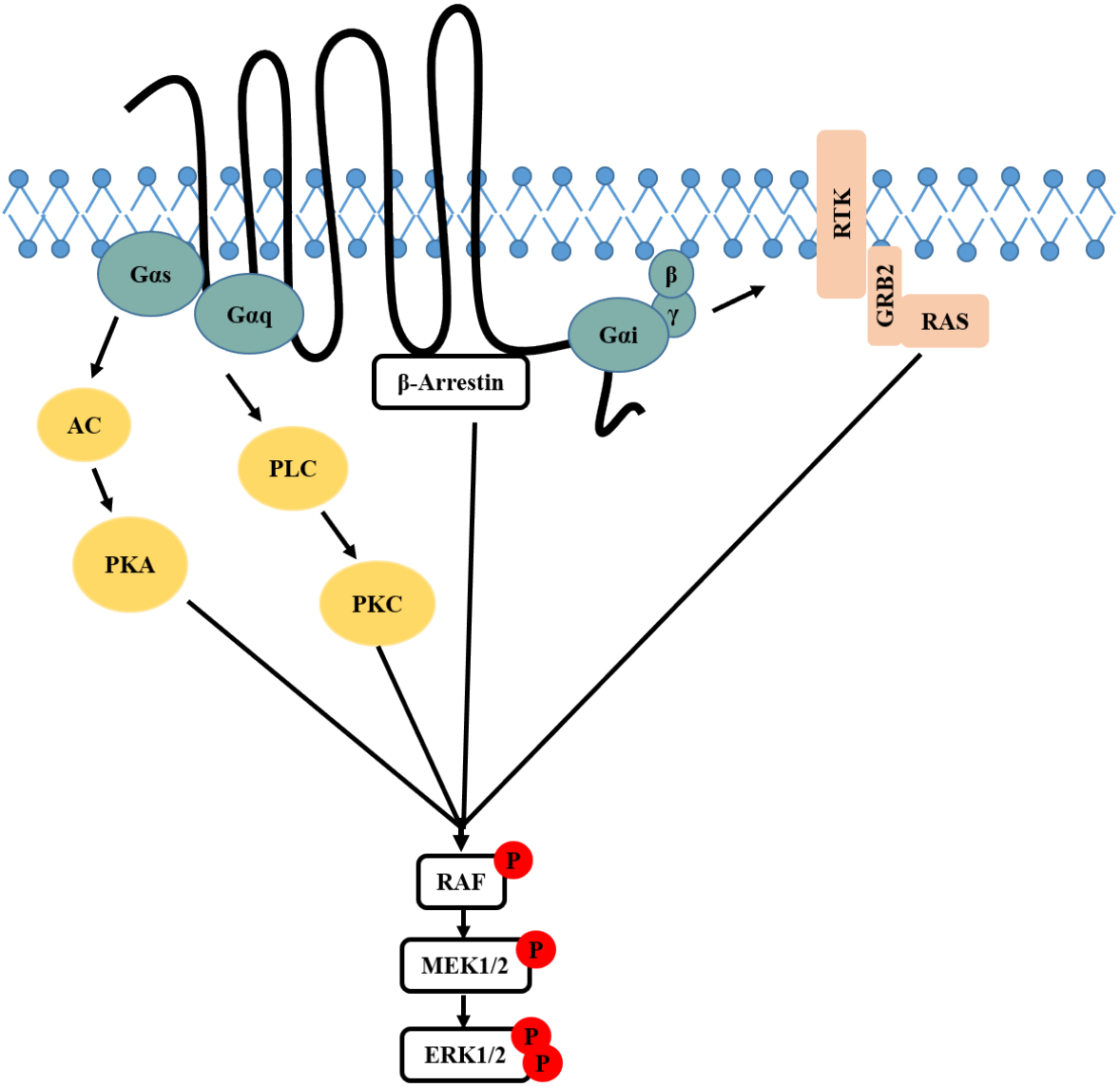

Figure 1.11. Representation of GPCR-mediated MAPK/ERK1/2 signaling pathway activation (Illustration reproduced courtesy of Cell Signaling Technology, Inc.).

In Figure 1.12 possible signaling pathways that were investigated in this thesis are illustrated.

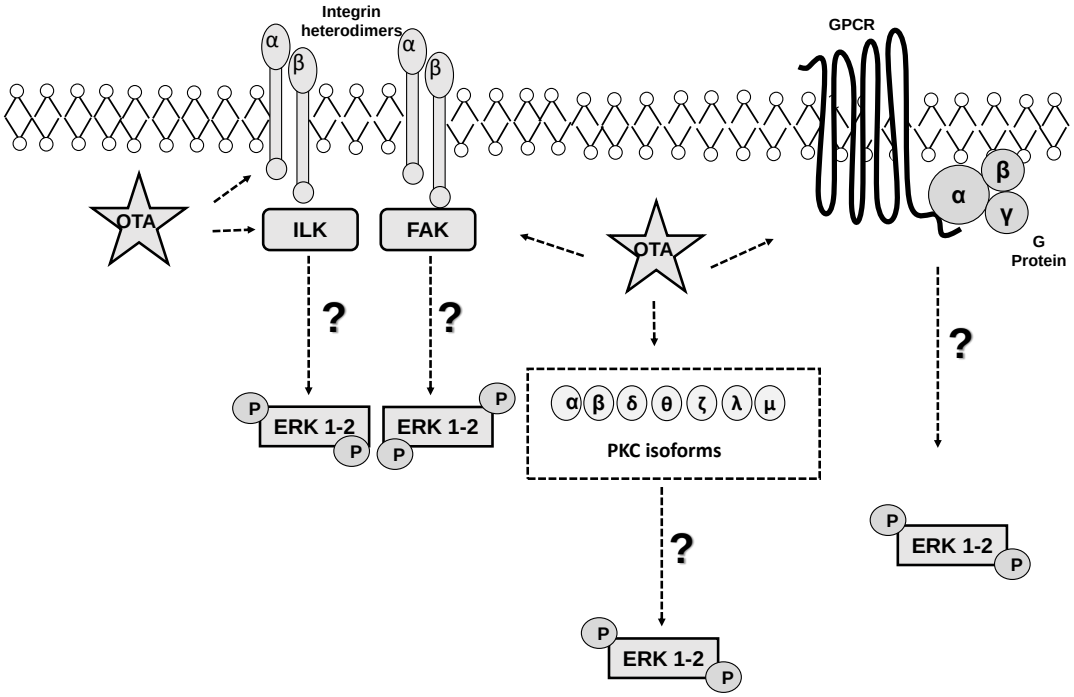

Figure 1.12. Candidate upstream signaling proteins in OTA-induced ERK1/2 activation

2. PURPOSE

OTA is an important nephrotoxic and carcinogenic mycotoxin posing danger for animal and human health. As it is a causative agent for kidney disease and seen as a potential carcinogenic agent, OTA has become very popular in toxicological research area. In our previous study with HK-2 cell line, it was reported that OTA causes sustained activation of MAPK/ERK1/2 pathways. OTA-induced apoptotic response was found to occur through ERK1/2 activation. Even though ERK1/2 pathway can be activated sustainably under OTA exposure, the upstream effectors playing key role in this activation are yet to be discovered.

Therefore, the aim of this study is to investigate and identify the upstream effectors of OTA-induced MAPK/ERK1/2 pathway in HK-2 cell line.

3. MATERIALS

3.1. Cell Line

Human kidney proximal tubular cell line (HK-2) was purchased from American Type Culture Collection (ATCC).

3.2. General Chemicals, Plastic and Glassware

All chemicals used in this study were purchased from Sigma Aldrich (USA), Cell Signaling (Beverly, Massachusets) and Roche (Switzerland) unless otherwise indicated in the text. All plastic and glasswares were purchased from TPP (Switzerland), Isolab (Germany), Sarstedt (Newton,USA), CAPP (Denmark) and they were all sterilized by autoclaving at 121°C for 20 minutes.

3.3. Cell Culture Chemicals and Reagents

Table 3.1: Chemicals and reagents used in cell culture.

Ochratoxin-A	Sigma-Aldrich, USA
EtOH	Sigma-Aldrich, USA
GF109203X	Santa Cruz, USA
Enzastaurin	Santa Cruz, USA
Gö6976	Santa Cruz, USA
Gö6983	Santa Cruz, USA
CTX	Sigma-Aldrich, USA
PTX	Sigma-Aldrich, USA
IBMX	Sigma-Aldrich, USA
ISO	The United States Pharmacopeial Convention USP, USA
H-89	Sigma-Aldrich, USA

Table 3.1: Chemicals and reagents used in cell culture (cont.).

Forskolin	Sigma-Aldrich, USA
8-bromo	Sigma-Aldrich, USA
Dulbecco's Modified Eagle Medium DMEM/F12 1:1)	PAN Biotech, Germany
Fetal Bovine Serum (FBS)	GibcoBRL, USA
0.25% Trypsin/0.913 mM EDTA, Phenol Red	GibcoBRL, USA
Penicillin-Streptomycin Solution	GibcoBRL, USA
PBS tablets	GibcoBRL, USA
PMSF	Roche, Germany
DMSO	AppliChem GmbH, Germany
TEMED	AppliChem GmbH, Germany
Acrylamide-bisacrylamide	AppliChem GmbH, Germany
Tween-20	AppliChem GmbH, Germany
Ammonium per sulfate (APS)	AppliChem GmbH, Germany
Tris-base	AppliChem GmbH, Germany
Glycine	AppliChem GmbH, Germany

3.4. Western blot chemicals

Table 3.2: Chemicals used in Western blot

4X Protein Loading Dye	8% (w/v) SDS 200mM TrisHCl pH 6.8 40% (w/v) 100% Glycerol 4% (w/v) β-mercaptoethanol 50 mM EDTA 0.08% (w/v) Bromophenol Blue
12% Resolving gel	375 mM TrisHCl pH 8.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide (12%/0.32% w/v) 0.05% (w/v) APS 0.005% (w/v) TEMED
Stacking Gel	0.125 mM TrisHCl pH 6.8 0.1% (w/v) SDS Acrylamide:Bisacrylamide (4%/0.1% w/v) 0.05% (w/v) APS 0.0075% (w/v) TEMED
Transfer Buffer	1% (w/v) Tris Base 14.4% (w/v) Glycine
TBS-T	50 mM TrisHCl pH 7.4 150 mM NaCl %0.05 Tween-20
Blocking Solution	5% (w/v) skim milk powder TBS-T
Primary Antibody Solution	5% (w/v)BSA 0.02% (w/v) Sodium Azide in TBS-T
Secondary Antibody Solution	5% (w/v) skim milk in TBS-T

3.5. Western Blot Reagents

Table 3.3. Chemicals used for protein extraction and quantification.

RIPA	150 mM NaCl 1% NP40 0.5% Sodium deoxycholate 0.1% SDS 50 mM Tris pH 7.4
Phosphatase Inhibitor Cocktail	Roche, Germany
Protease Inhibitor Cocktail	Roche, Germany
Bovine Serum Albumin (BSA)	Thermo Scientific,USA
Protein-DC Assay Kit	Bio-RAD, USA

3.6. Western Blot Antibodies

Antibodies were purchased from Cell Signaling (Beverly, Massachusets).

Table 3.4: Antibody hosts and concentrations

Primary Antibody	**Host**	**Dilution**
p44/42 MAPK (ERK1/2)	Rabbit	1:1000
p-p44/42 MAPK (ERK1/2)	Rabbit	1:1000
ILK	Rabbit	1:1000
Integrin $\alpha 4$	Rabbit	1:1000
Integrin $\alpha 5$	Rabbit	1:1000
Integrin αV	Rabbit	1:1000
Integrin $\beta 1$	Rabbit	1:1000
Integrin $\beta 3$	Rabbit	1:1000
Integrin $\beta 4$	Rabbit	1:1000
Integrin $\beta 5$	Rabbit	1:1000
FAK	Rabbit	1:1000
FAK Y397	Rabbit	1:1000
FAK Y925	Rabbit	1:1000
FAK Y576/577	Rabbit	1:1000
PKD-PKCμ	Rabbit	1:1000
p-PKD-PKCμ Ser916	Rabbit	1:1000

Table 3.4: Antibody hosts and concentrations (cont.).

p-PKD-PKCμ Ser744/748	Rabbit	1:1000
PKC panβII Ser660	Rabbit	1:1000
Phospho-PKCα/β Thr638/641	Rabbit	1:1000
Phosho-PKCδ Thr505	Rabbit	1:1000
Phosho-PKCδ/θ Ser643/676	Rabbit	1:1000
Phosho-PKCθ Thr538	Rabbit	1:1000
Phosho-PKCζ/λ Thr410/403	Rabbit	1:1000
B-actin	Rabbit	1:1000
Rabbit IgG	Goat	1:2000

3.7. Special Kits

Table 3.5. List of kits used in this study.

Protein DC Assay Kit	BioRad
Caspase-Glo® 3/7 Assay Systems	Promega, USA
cAMP ELISA Kit	Cell Signaling

3.8. Equipments

Table 3.6: List of equipments used for this study.

4°C Room	Birikim Elektrik, Turkey
Autoclaves	Astell Scientific, UK
Centrifuge	Beckman Coulter J-MC superspeed centrifuge
Western blot equipments	Bio-Rad Labs, USA
Refrigerator (4°C)	Ugur, USS 300 DTK, Turkey
Deep Freezers (-20°C)	Ugur, UFR 370 SD, Turkey
Deep Freezers (-80°C)	Sanyo Ultra Low, UK,Thermo Scientific, USA
Deep Freezers (-150°C)	Sanyo MDF-1156, UK,Thermo Scientific, USA

Table 3.6: List of equipments used for this study (cont.).

Stella Raytest	Germany
Vortex	Scientific Industries, USA
CO2 incubator	MCO-18AC, Sanyo, Japan
CO2 Tank	Genç Karbon, Turkey
Dish washer	Mielabor G7783, Miele, Germany
Laminar flow cabinet	Class II B Tezsan, Turkey
Ice Machine	Scotsman Inc. AF20, Italy
Autoclaves	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Heat blocks	DRI-Block DB-2A, Techne, UK
Microplate Reader	680, Bio-Rad, USA
Oven	Gallenkamp 300, UK
pH Meter	WTW, Germany
Pipettes	Axygen, USA
Pipettor	Brandtech Accu-jet, USA
Power Supply	Power Pac Universal, Bio-Rad, USA
SDS-PAGE Transfer System	Bio-Rad, USA
Vortex	VWR, USA
Water purification	WA-TECH UP Water Purification Sys. Germany
Luminometer	Fluoroskan AscentTM FL, Thermo Scientific, USA
Magnetic Stirrer	MS-H-S, Dragonlab, China

4. METHODS

4.1. Cell Culture Tecniques

4.1.1. Thawing of Cells

Sufficient number of the vials of frozen cells were taken from -150°C freezer and immediately thawed at 37°C dry block heater (DRI-Block DB-2A, Techne, UK). After cells were melted, they were transferred to 15 ml falcon tubes and 1 ml fresh growth medium was added for each vial. The cells were precipitated at 300g for 5 minutes. Supernatant was discarded and the pellet was resuspended in growth medium. Finally the suspended cells were transferred to fresh cell culture plates.

4.1.2. Growth and Treatment Conditions of Cells

HK-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12 supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100μg/ml streptomycin. Cells were treated with chemicals in the assay medium (Dulbecco's modified Eagle's medium (DMEM) F-12 supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and100μg/ml streptomycin). The cells were incubated in humidified incubator at % 5 CO_2 and 37°C.

4.1.3. Passaging of Cells

When cells became confluent on petri dish the medium was aspirated and the cells were washed once with 1X PBS. To remove the cells from the culture plate, trypsin (0.025%, ready-to-use) was added on plate and the cells were incubated at 37°C for 2-3 minutes. Eight ml of fresh growth medium was added to inactivate the trypsin. Then, cells were transferred to falcon tubes and vortexed gently in order to disperse the cells. After that the cells were centrifuged at 300 g for 5 minutes and the supernatant was discarded. The cells were resuspended in fresh growth medium and then transferred

to cell culture plate.

4.1.4. Cryopreservation

After cells covered the culture plate, the medium was aspirated and the cells were washed once wit 1X phosphate buffer saline (PBS). Then they were trypsinized for 2-3 minutes at 37°C. The detached cells were collected in a falcon tube and pelleted at 300g for 5 minutes. Supernatant was discarded and the cells were resuspended in FBS. Then Dimethyl Sulfoxide (DMSO) was added to be the 10% of the total volume. The cells were distributed into cryovial tubes as 1ml for each of tubes. The cryovials were incubated at -80°C overnight and then transferred to -150°C freezer.

4.2. Treatment Conditions of HK-2 Cells

HK-2 cells were seeded at a density of $2x10^6$ on 10-cm cell culture plate in assay medium unless indicated otherwise. After 24-hour of seeding, the cells were treated either with 10μM of OTA or 0.1% vehicle (EtOH). Then the cells were incubated in OTA and vehicle for a desired time period.

4.2.1. PKC Inhibitor Treatment for Western blot

HK-2 cells were seeded at a density of $2x10^6$ on 10-cm cell culture plate in assay medium unless indicated otherwise. After 24-hour of seeding, the cells were treated either with 10μM of OTA, 10μM of inhibitors (GF109203X, Enzastaurin, Gö6976, Gö6983), or 0.1% EtOH and DMSO as vehicles. Then the cells were incubated in OTA and vehicle in a desired time.

4.2.2. cAMP Agent Treatment for Western Blot

HK-2 cells were seeded at a density of $2x10^6$ on 10-cm cell culture plate in assay medium unless indicated otherwise. After 24-hour of incubation, the cells were treated either with 10μM of OTA, Forskolin, IBMX, ISO and H-89, 100μM 8-bromo, or 0.1% EtOH and DMSO. Then the cells were incubated in OTA and vehicle in a desired time.

4.3. Western Blotting

4.3.1. Cell Lysis and Protein Extraction

Cells were treated with chemicals on 10-cm cell culture plate. The medium was aspirated and the cells were washed once with 1X PBS. As soon as the cells were washed, 400of RIPA buffer was added onto the cells and then they were put on ice immediately. After incubated for 5 minutes on ice the cells were scraped by using cell lifters. Lysates were homogenized by passing through 10ml-gauge syringe six times. Then the lysates were centrifuged at 14000xg for 15 minutes at 4°C. Finally the supernatant was transferred into a 1.5ml eppendorf tube.

4.3.2. Quantification of Protein Lysates

Protein concentrations were measured by following protocol provided by Protein DC Assay Kit (BioRad, USA). BSA stock, which was supplied with the kit, was serially diluted to 4mg/ml, 2mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml in 1X PBS. Reagent A'' working solution was prepared by mixing Reagent A and Reagent S in 1:50 ratio. 0.5ml eppendorf tubes were prepared as the same number of standard and samples. And after each tube was labeled, 75μl from the Reagent A$'$ was distributed to the tubes. For each 15μl standard volume, 3μl from the sample was added to the tubes. So, the samples were diluted as 1:5 ratio. In order to make triplicate measurement, prepared solutions were added to 96-well plate three times as 25μl/well. Then 200μl Reagent B was added into each well. 96-well plate was incubated at room temperature for 15 minutes and the absorbance was measured at 750nm.

4.3.3. Preparation of samples for SDS gel page

In order to prepare the samples, the concentration of each sample was equalized to a fixed concentration by using from sample and RIPA buffer as required amount. Finally, 4X loading dye (LD) was added to samples.

4.3.4. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10resolving and 4stacking polyacrylamide gels were prepared for loading of samples. After polymerization of the gels, the samples were boiled at 95°C for 5 minutes and then the samples were loaded onto gel with 5μl of prestained protein ladder (PageRulerTM, (Thermosicentific) and run in 1X SDS buffer at 80V until the samples reached the resolving gel. Following this, the voltage was increased to 100V and the samples carried on running until the bromophenol blue reached the end of the gels. After the proteins were separated they were transferred to PVDF membrane (Millipore, 0.22μm)with Bio-Rad semi-dry transfer system. PVDF membrane and Watmann paper (Thermo Scientific,USA) were cut according to the size of the gel. In order to activate the membrane, it was immersed in absolute methanol and then water. The activated membrane, Watmann papers and gels were immersed in the transfer buffer. Afterward, the membrane and the gel were inserted between two Watmann papers and then they were put onto the transfer cassettes. Transfer was performed at 100V for 90 minutes. When the transfer was over, membranes were washed in 1X TBS-T once for 3 minutes and then the membrane was incubated in blocking solution for 2 hours. Following this, membranes were washed three times in TBS-T each for 5 minutes and the membranes were incubated in primary antibody overnight at 4°C. The next day, membranes were washed three times in TBS-T each for 5 minutes and the membranes now were incubated in secondary antibody for 2 hours at room temperature. When incubation in antibody is over, the membranes were washed again three times in TBS-T. Then the membranes were incubated in chemiluminescent substrate (SuperSignalTM, West Pico, Thermoscientific) for 1 minute and then the chemiluminescence signal was captured using Stella digital bioimaging system (Raytest). The band intensities were analyzed using imageJ.

4.4. Cyclic AMP Chemiluminescent Assay

Cell Signaling Technologies Cyclic AMP XP® Chemiluminescent Assay Kit instructions was followed in order to perform chemiluminescence measurement. This assay kit principle depends on the competition enzyme-linked immunoassay used to determine cAMP levels on cells. In this assay, cAMP in the experiment samples competes with a fixed amount of HRP-linked cAMP in order to bind to an anti-cAMP XP® rabbit monoclonal antibody immobilized onto a 96-well plate. Right after washing the excess amount of cAMP and HRP-linked cAMP, chemiluminescent reagent provided in the kit is added for signal progression. Since the kit principle depends on a competition between the HRP-linked cAMP and intracellular cAMP the magnitude of light emission is inversely proportional to the quantity of intracellular cAMP (Figure 4.1. cAMP standard is also provided with this kit, thus the user has an opportunity to calculate the absolute amount of cAMP in a sample of interest.

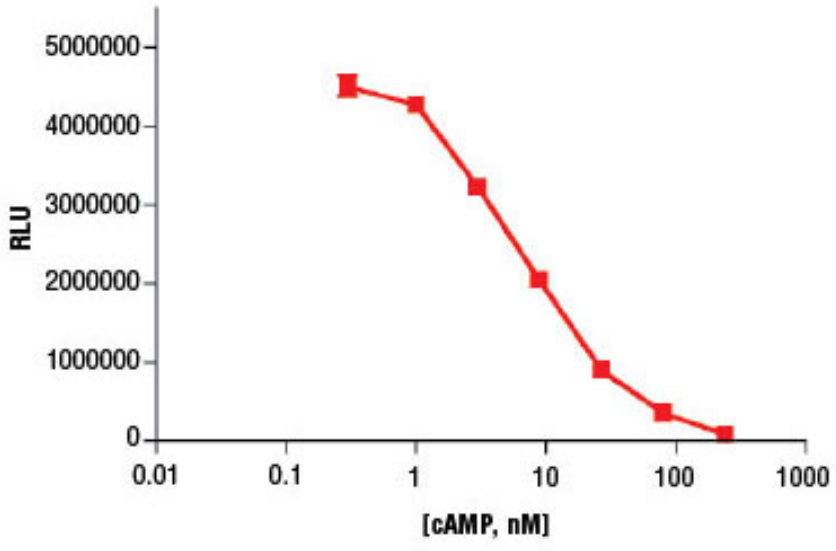

Figure 4.1. cAMP standards assayed with Cell signaling Cyclic AMP XP®. The more the concentration of cAMP increase the more the signal decreases.

The cells were seeded at a density of 7,5x10^3 cells/100μl per well on 96-well tissue culture plate in assay medium. After an overnight incubation, the cells were treated either with chemicals or the vehicle and then incubated for the desired time period. The cells were rinsed twice with 200μl ice cold PBS, and then 100μl/well 1X lysis buffer. The cells were kept on ice for 5 to 10 minutes. All kit components were brought to room temperature. cAMP standards were prepared in the 1X cell lysis buffer. Twenty-five μl of the HRP-linked cAMP solution and 25μl sample were added to the wells of cAMP plate. The plate was covered and incubated at room temperature for 3 hours on a horizontal orbital plate shaker. The plate contents were discarded and wells were washed four times with 200μl/well of 1X wash buffer. Working solution was prepared by mixing equal parts of Luminol/Enhancer Solution and stable peroxide buffer. Then 50μl of working solution was added to each well.

Plate-based luminometer (Thermo Scientific, Fluroskan Ascent Fl) was used to measure Relative Light Units (RLU) at 425nM with 1-10 minutes following addition of the substrate.

4.5. Caspase 3/7 Glo® Assay

Caspase-Glo® 3/7 Assay (Promega) protocol was performed in order to measure the caspase activity in HK-2 cell line. Caspase-Glo® 3/7 Assay is a luminescent assay depending on the measurement of caspase-3 and caspase-7 activities. Caspase family refers to "cysteine aspartic acid-specific protease" and this family plays a vital role in apoptotic pathway in mammalian cells. This assay provides luminogenic caspase 3/7substrate, which is DEVD (Asp-Glu-Val-Asp) containing tetrapeptit sequence. Upon adding Caspase-Glo® 3/7 Assay reagent in a mixture results in cell lysis and subsequently DEVD is cleaved by the caspases released from the lysed cells. This cleavage results into the release of aminoluciferin that is another substrate for luciferase enzyme. Assay is completed with a glow-type luminescent signal generated by the luciferase activity. Assay principle is represented in figure 4.2.

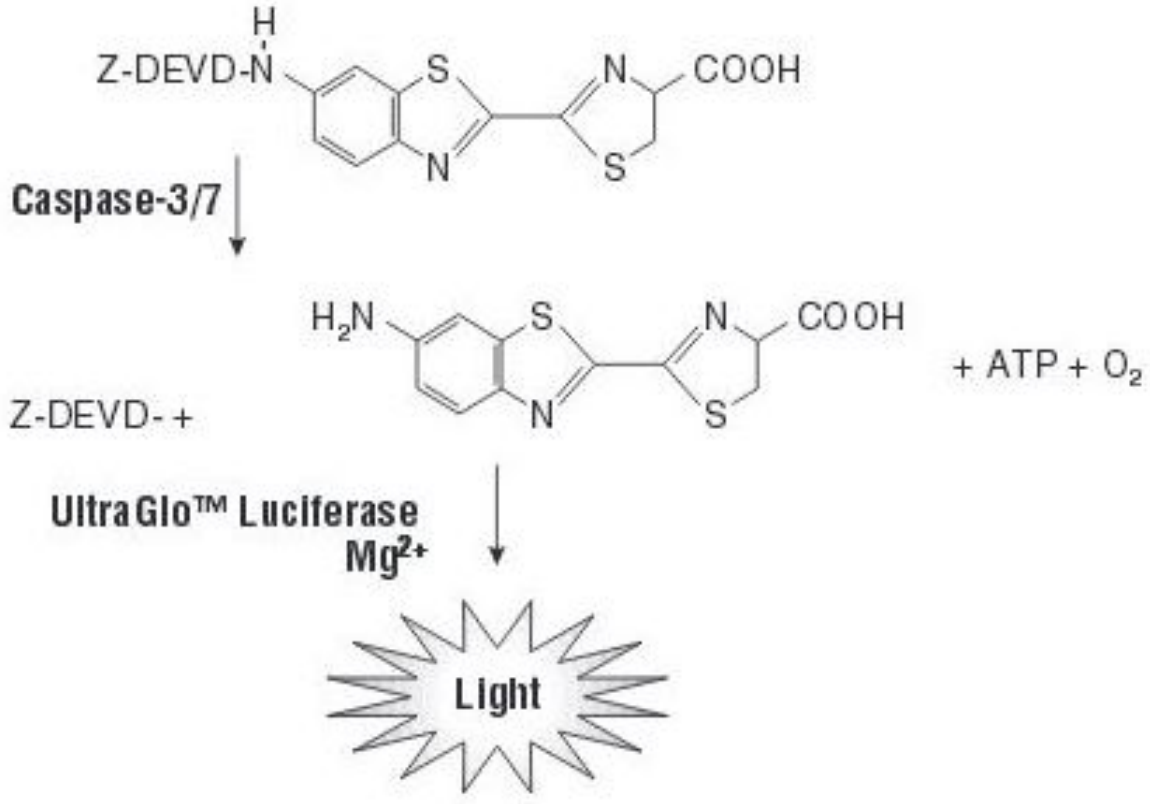

Figure 4.2. Schematic representation of Caspase-Glo® 3/7 Assay working principle.

HK-2 cells were seeded at a density of 7.5x10/100μl per well on white 96-well tissue culture plates designed for luminescence measurement in assay medium. The cells were treated either with 10μM OTA, vehicle (EtOH and DMSO), or 10μM cAMP adjuvant chemicals (3-isobutyl-1-methylxanthine (IBMX) and Forskolin, 100μM cAMP analog (8-Bromoadenosine 3',5'-cyclic monophosphate), and 10μM cAMP-dependent kinase inhibitor (H-89). cAMP modulating agents were added 1 hour prior to OTA. 1X concentration of agents were prepared in 3ml assay medium. Assay medium from the day before was aspirated and 50μl of new 1X medium containing the chemical agent was added to each well. The cells were incubated at 37°C for 1 hour. After that, 2X concentration of OTA the cells were prepared in 3ml assay medium and then 50μl from 2X medium was added on each well. Cells were incubated for 6 and 24 hours at 37°C. When the incubations ended, the plates were incubated outside of the incubator for 15 minutes in order to reach to room temperature. To the each well, 100μl of Caspase-Glo® 3/7 Reagent was added and then the plate was centrifuged at 300 rpm for 30 seconds. Then the plate was further incubated at room temperature for two hours and finally luminescence measurements were taken.

4.6. Statistical Analysis

Results were obtained from three separate experiments. Quantitative data were analyzed using GraphPad Prism v5.04. The data were presented as mean ± SEM or SD and the significance was tested by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test where needed. "*" denotes statistical significance (*, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$).

5. RESULTS

In this project, the possible upstream effectors of OTA-induced sustained ERK1/2 activation in HK-2 cells were investigated. It is already known that OTA has the ability to bind to serum albumin and this binding reduces OTA activity [10]. For this reason, 10μM OTA concentration and 5% FBS in the media were decided based on viability and proliferation assays performed in our lab.

5.1. ERK1/2 activation under OTA treatment at different time points in HK-2 cells.

In order to identify upstream effectors of ERK1/2 activation, we started by confirming our previous results showing ERK1/2 activation initiated at early time points of OTA treatment and sustained up to 24 hours in HK-2 cells. We observed that in HK-2 cells treated with 10μM OTA, ERK1/2 activation began at early time points of treatment and sustainably continued up to 24 hours by Western blot analysis. The most significant increase in the ERK1/2 activity was observed at third and sixth hours of treatment confirming our previous results (Figure 5.1).

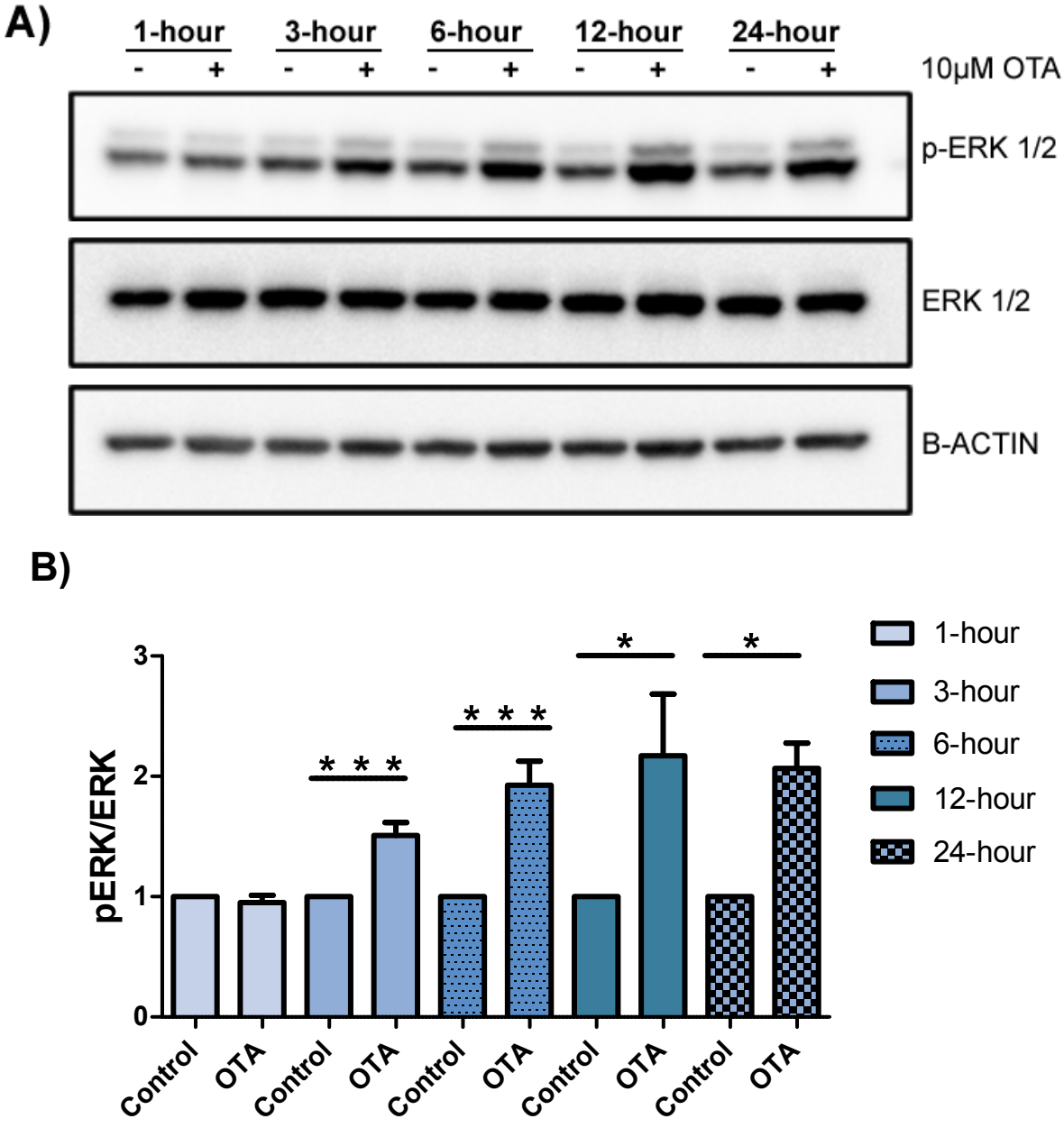

Figure 5.1. Effect of OTA treatment on the activation of ERK1/2 in HK-2 cell line. A) Protein extracts of HK-2 cells treated with 10μM OTA or 0.1% EtOH as vehicle. B) Densitometric analysis of p-ERK1/2 activation is shown was performed after normalization with ERK1/2. Blots shown are representative of Western blot analysis from three separate experiments.

5.2. Expression levels of Integrin chains and ILK proteins in HK-2 cells under OTA treatment at different time points.

Integrins and Integrin-linked Kinases (ILKs) were the first upstream effector candidates for sustained ERK1/2 activation in OTA-treated HK-2 cell line on the basis of proteomics data. It is well known that integrins are found at plasma membrane as heterodimer of α- and β- subunits [48]. $\alpha5\beta1$ and $\alpha V\beta3$ are the most common heterodimers found in plasma membrane and display their activity [99, 100]. In addition, it is already known that integrins require ILK to relay their activity to targeted proteins in the signaling cascade [49]. Therefore, the expression levels of ILK, $\alpha5$, $\beta1$, αV and $\beta3$ were examined together via Western blotting. It was determined that expression level of ILK was higher in OTA-treated HK-2 cell line compared with control groups in the first hour of treatment. For further time points of OTA treatment, it was observed that expression level of ILK did not change. On the other hand, it was demonstrated that expression levels of $\alpha5$ and $\beta1$ subunits increased in the first hour of OTA exposure. However, the increase was not sustained. The increase returned to the basal levels by the third hour of experiment for both subunits. And in the sixth and 12^{th} hours of experiment, the expression of $\alpha5$ subunit slightly increased. Concordantly, expression level of $\beta1$ subunit was observed to increase at sixth and 12^{th} hours of experiment. Expression levels of Integrin αV did not change under OTA exposure in HK-2 cells. Integrin $\beta3$ subunit expression levels were increased at all time points except for 24^{th} hour, and the highest expression levels were seen at the first hour of OTA treatment (Figure 5.2).

Beside the former ones, integrin $\alpha4$, $\beta4$, and $\beta5$ expression levels were also examined. Integrin 4 demonstrated a tendency to decrease in OTA-exposed HK-2 cells compared with the control cells. Integrin $\beta4$ was observed to have almost the same pattern with $\alpha4$, showing a tendency to decrease in OTA-treated HK-2 cells. Expression levels of $\beta5$ were lower in OTA-treated cells compared with control group except for 24^{th} hour of the experiment. At 24^{th} hour of the experiment $\beta5$ again was observed to increase in OTA-exposed HK-2 cells (Figure 5.3).

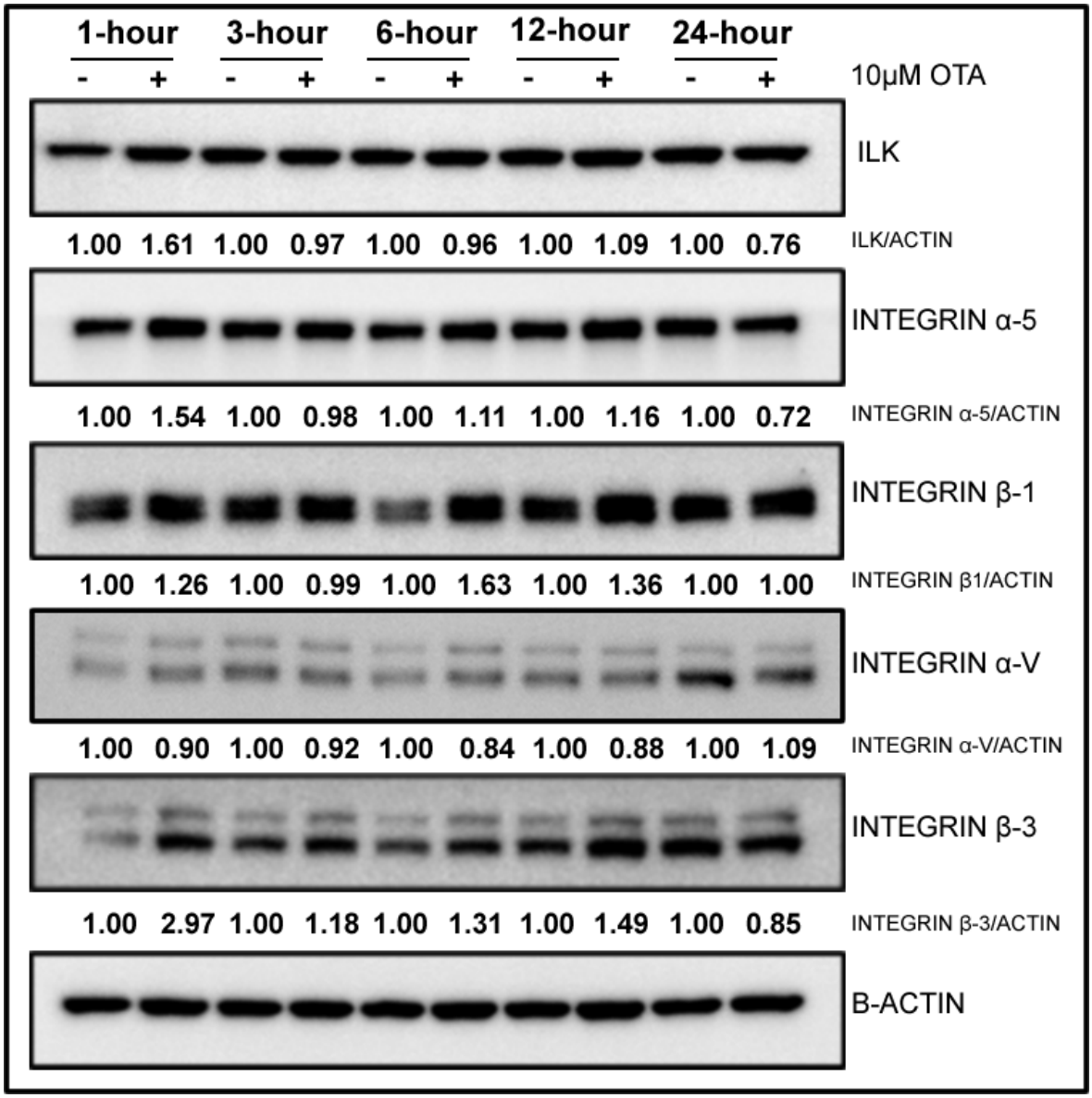

Figure 5.2. Effect of OTA treatment on the expression level of ILK and Integrin subunits in HK-2 cell line. Cells were treated either with 10 μM OTA or 0.1% EtOH as vehicle for indicated time points. Relative expression levels of indicated proteins in OTA-treated samples (normalized to actin) are shown with their ratios to the corresponding control group.

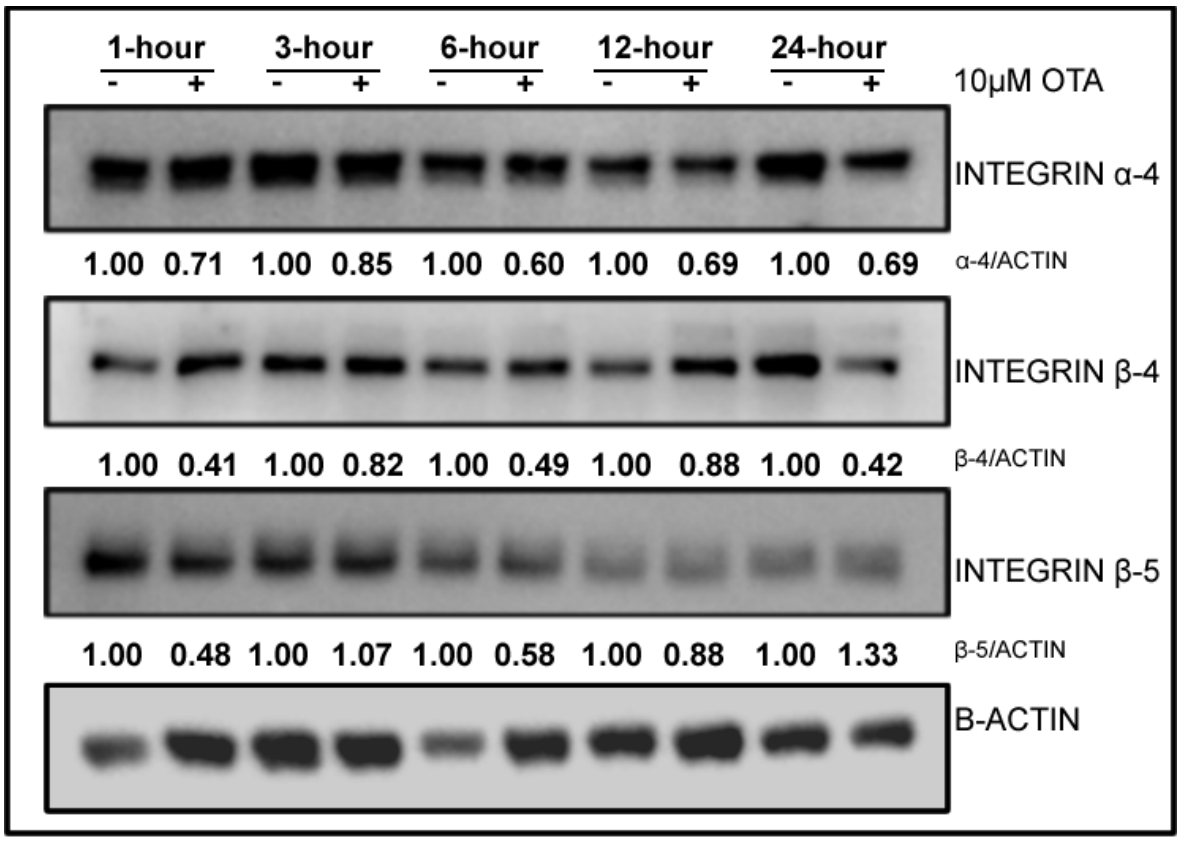

Figure 5.3. Effect of OTA treatment on the expression levels of other Integrin subunits in HK-2 cell line in time-dependent manner. Cells were treated either with 10μM OTA or 0.1%EtOH as vehicle for indicated time points. Relative expression levels of indicated proteins in OTA-treated samples (normalized to actin) are shown with their ratios to the corresponding control group.

5.3. Activation change of Focal Adhesion Kinase (FAK) proteins in HK-2 cell line under OTA exposure at different time points.

For sustained activation of MAPK/ERK1/2 pathway, Focal Adhesion Kinase (FAK) was another candidate protein kinase that might be responsible as an upstream effector in OTA treated HK-2 cell line. FAK is known to be activated by integrin clustering on the plasma membrane and the clustering of integrins results in autophosphorylation of FAK at Tyr397 residue that is a binding site for other kinases such as PLC, PI3K, and Src family kinase[65]. Recruitment of Src family kinases leads to the phosphorylation of Tyr576 and Tyr577 in the catalytic domain and Tyr925 in the carboxy-terminal region of FAK [64]. Because Tyr576 and 577 residues lie in the activation loop of the kinase domain, the phosphorylation levels of these residues might have implications in catalytic activity of FAK protein under OTA exposure. Since there was increase at early time points in the expression levels of some integrin subunits such as integrin $5\beta1$ and integrin V$\beta3$, it is possible that the clustering of integrins could lead to activation of FAK protein. Unfortunately, OTA treatment in HK-2 cells did not have an activity effect on Tyr397 at any hours of treatment. This situation was almost the same for Tyr925 residue except for a very slight increase at first hour of treatment. For Y576/577 residues, there was a slight increase in the phosphorylation at the first hour of OTA treatment. However, this activation was not seen in the late hours of treatment up to 24-hour (Figure 5.4).

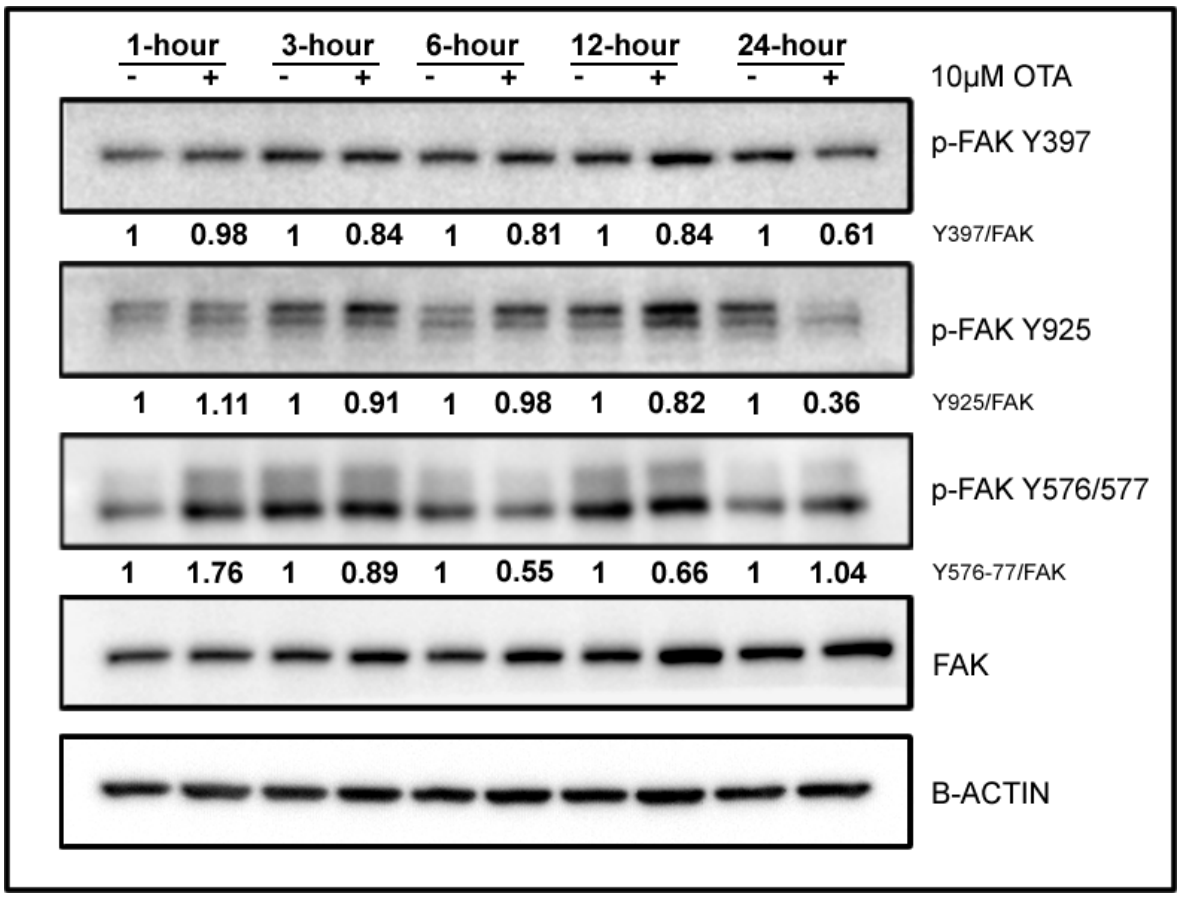

Figure 5.4. Effect of OTA treatment on the phosphorylation of FAK residues in HK-2 cell line. Cells were treated either with 10μM OTA or 0.1% EtOH as vehicle for indicated time points. Relative expression levels of indicated proteins in OTA-treated samples (normalized to FAK) are shown with their ratios to the corresponding control group

5.4. Activation change of Protein Kinase C (PKC) in HK-2 cell line exposed to OTA at different time points.

Due to the fact that there are more than one isoform of PKC, HK-2 cell line under OTA exposure at different time points was analyzed in terms of activity change in PKC isoforms through Western blotting analysis. In conventional isoforms of PKC enzyme, OTA did not cause a significant activation in HK-2 cell line. A slight increase was observed in the phosphorylation of β isoforms of conventional PKC at 24^{th} hours of OTA treatment (Figure 5.5). On the other hand, OTA treatment resulted with the activation of novel isoform δ on T505 residue at third hour of treatment. PKC θ isoform was observed to be active on T538 residue at 24^{th} hour of OTA exposure. (Figure 5.6). Atypical isoforms of PKC were not observed to be activated by OTA exposure in HK-2

cell line (Figure 5.7). PKCμ isoform is categorized as novel atypical isoforms of PKC enzymes [101]. HK-2 cells which were treated with OTA showed increase activation of Ser916 and Ser744/748 residues of PKCμ enzyme (Figure 5.8).

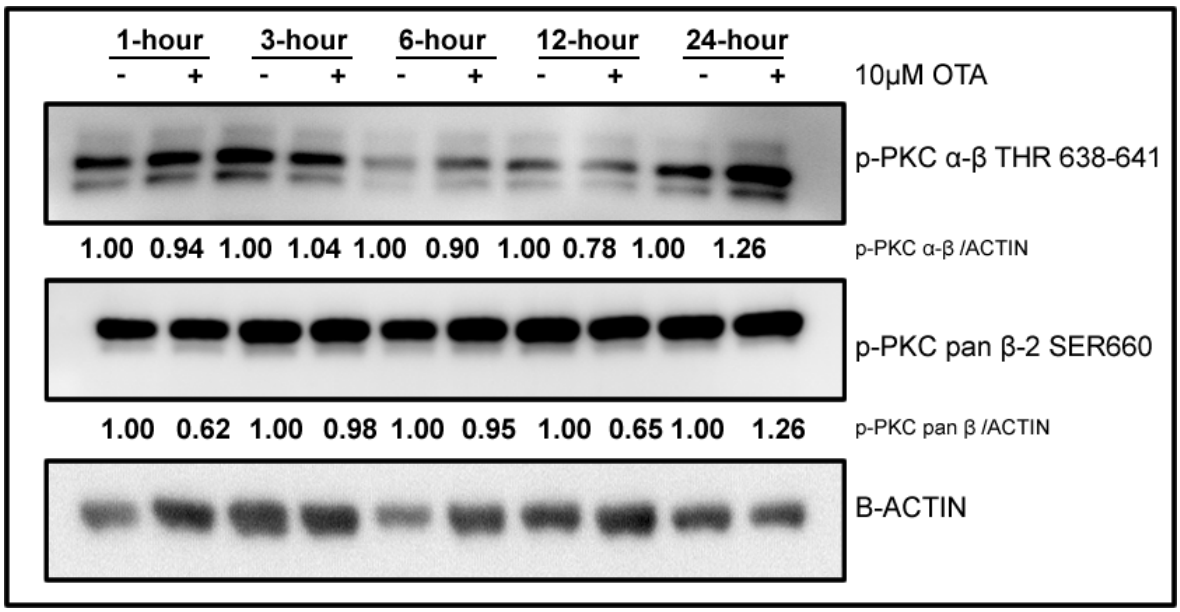

Figure 5.5. Effect of OTA treatment on the activation of the conventional isoforms of PKC enzyme in HK-2 cell line. Cells were treated either with 10μM OTA or 0.1% EtOH for indicated time points. Relative expression levels of indicated proteins in OTA-treated samples (normalized to actin) are shown with their ratios to the corresponding control group.

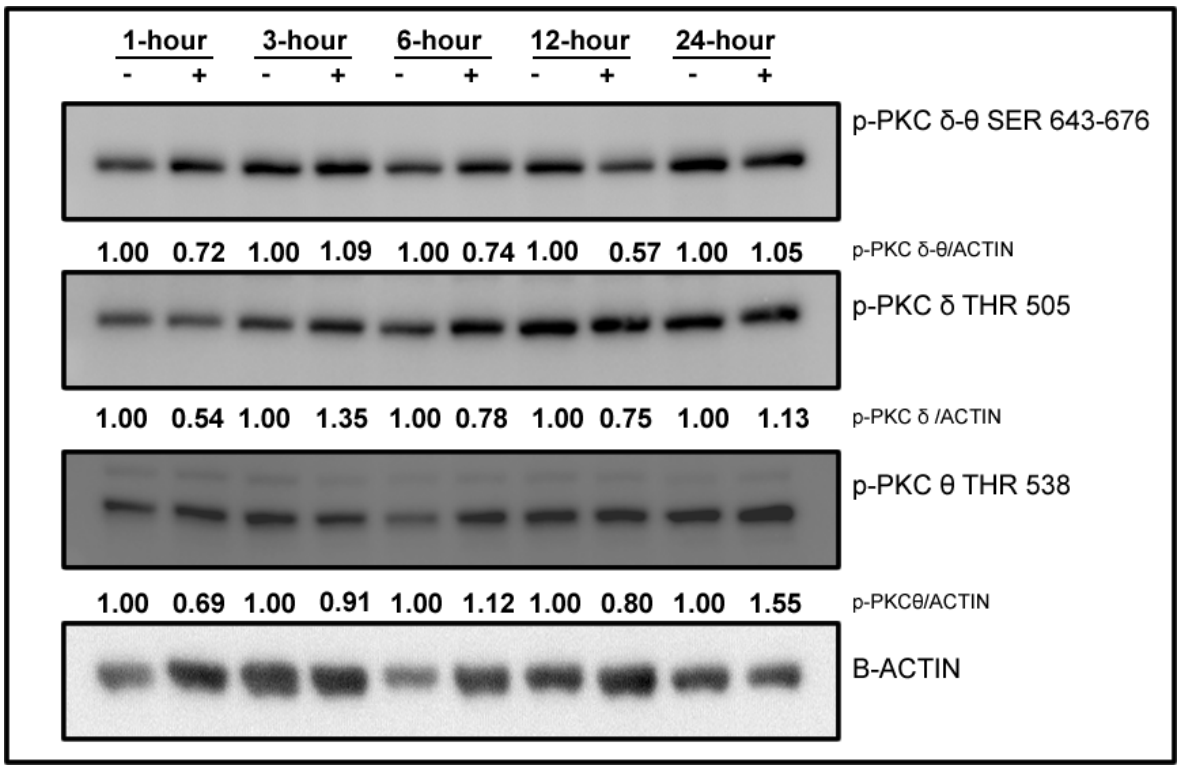

Figure 5.6. Effect of OTA treatment on the activation of the novel isoforms of PKC enzyme in HK-2 cell line. Cells were treated either with 10μM OTA or 0.1% EtOH for indicated time points. Relative expression levels of indicated proteins in OTA-treated samples (normalized to actin) are shown with their ratios to the corresponding control group.

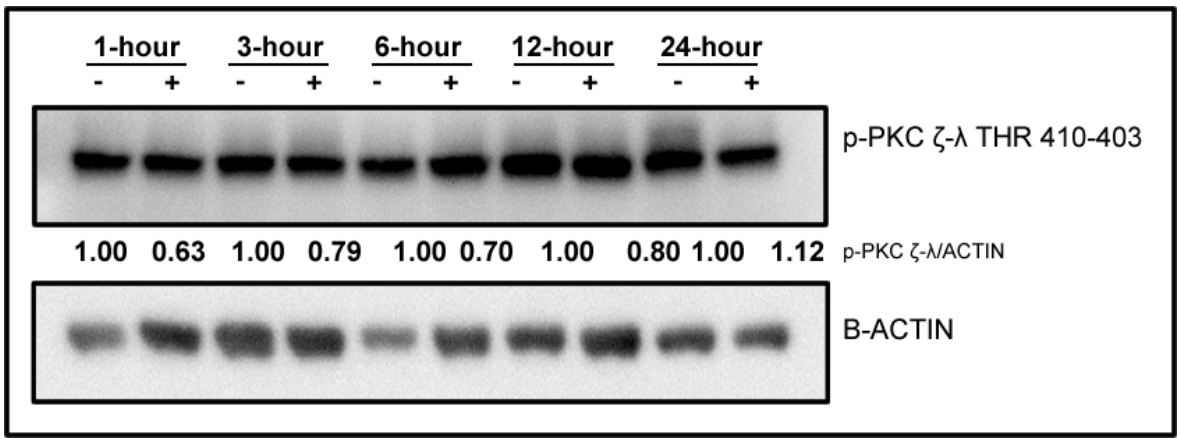

Figure 5.7. Effect of OTA treatment on the activation of the atypical isoforms of PKC enzyme in HK-2 cell line. Cells were treated either with 10μM OTA or 0.1% EtOH for indicated time points. Relative expression levels of indicated proteins in OTA-treated samples (normalized to actin) are shown with their ratios to the corresponding control group.

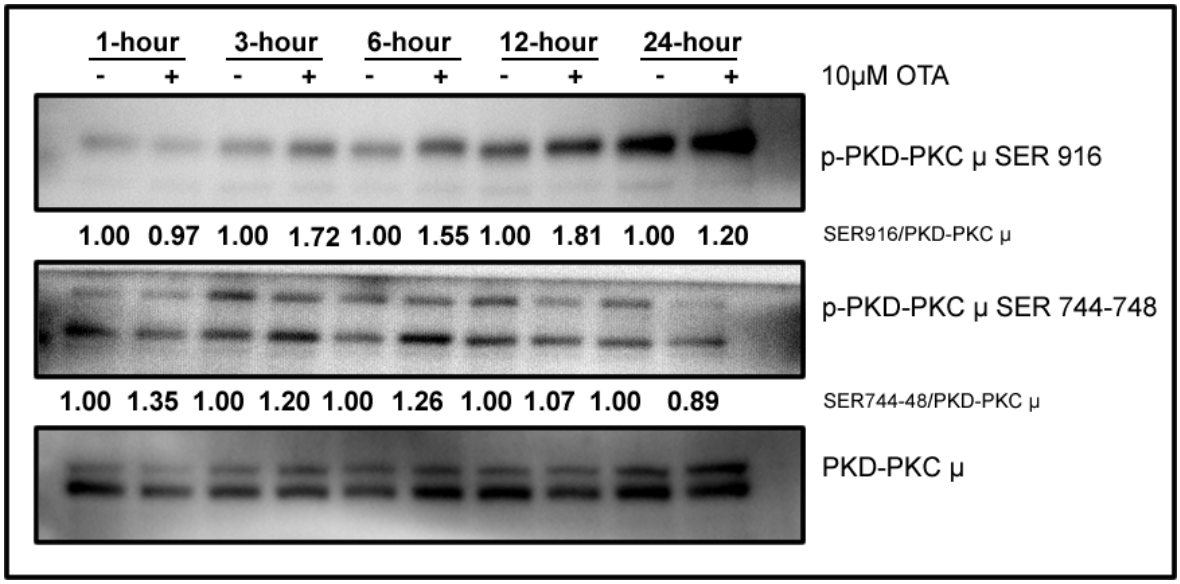

Figure 5.8. Effect of OTA treatment on the activation of PKD-PKCμ enzyme in HK-2 cell line. Cells were treated either with 10μM OTA or 0.1% EtOH for indicated time points. Relative expression levels of indicated proteins in OTA-treated samples (normalized to PKD-PKCμ) are shown with their ratios to the corresponding control group.

5.5. Effect of PKC enzyme inhibition on the phosphorylation levels of PKC isoforms and p-ERK1/2 activation

We wanted to observe whether inhibition of PKC enzymes cause an attenuation in ERK1/2 activation in HK-2 cells. We inhibited isoforms which were activated under OTA exposure. Four types of chemicals were used in order to inhibit different PKC isoforms. These are GF109203X, Enzastaurin, Gö6976, and Gö6983.

GF109203X also known as Bisindolylmaleimide I is the most common PKC inhibitor that is specific to certain isoforms of PKC. It shows its inhibitory effect on PKCα, PKCβI, PKCβII, and PKC with high selectivity [102, 103].

Enzastaurin also named as LY317615 has been known as PKCβ, PKCα, PKCγ, and PKCϵ isoforms inhibitor [104, 105].

Gö6976 has been known to be an effective inhibitor of PKC especially PKCα and PKCβ1 isoforms. It has also been shown as a potent inhibitor for Janus Kinase 2 (JAK2) and Fms-like tyrosine kinase 3 (Flt3) [106, 107].

Gö6983 has inhibitory role against for PKCα, PKCβ, PKCγ, and PKC isoforms. However, it is a poor inhibitor to be used against PKCζ and PKCμ [108].

Based on the literature survey, it was decided to treat the HK-2 cells with 10μM concentration of these inhibitors alone or in combination with OTA for three hours. Then, the phosphorylation levels of PKC isoforms and ERK1/2 proteins were analyzed by utilizing Western blotting. In agreement with the previous results, ERK1/2 phosphorylation and therefore activation was increased at 3 hours of OTA treatment (Figure 5.9). Unexpectedly, it was clearly observed that ERK1/2 activation was enhanced by the addition of GF109203X and Enzastaurin in OTA-treated HK-2 cells. On the other hand, Gö6976 and Gö6983 chemicals were determined to have inverse effect on the ERK1/2 activation induced by OTA (Figure 5.9). Gö6983 inhibited novel PKC isoform δ Thr505 (Figure 5.10). Gö6983 could also inhibit Ser916 residue of PKCμ isoform (Figure 5.11). These two isoforms were activated by OTA treatment in HK-2 cells (Figure 5.6 and 5.8).

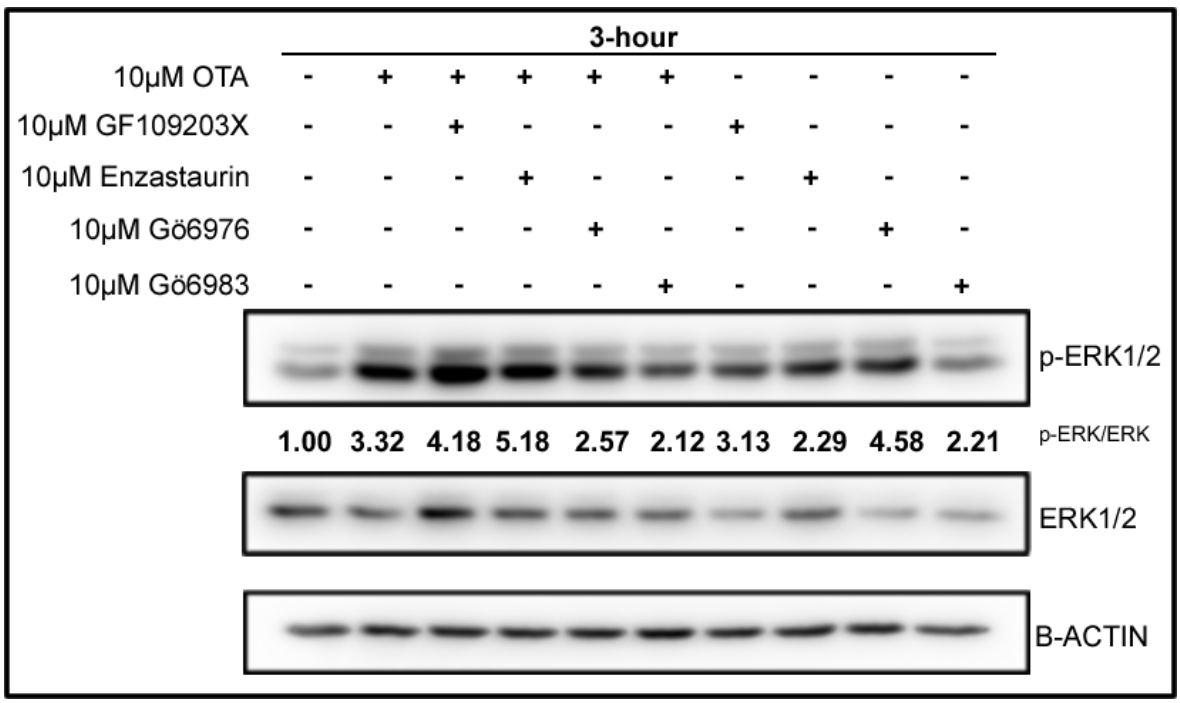

Figure 5.9. Effects of PKC inhibitors on ERK activity induced by OTA. Cells were treated either with 10μM OTA alone or in combination with10μM GF109203X, 10μM Enzastaurin, 10μM Gö6976, and 10μM Gö6983 respectively for indicated time points. 0.1% EtOH and DMSO were used as vehicles. Inhibitors were given to HK-2 cells 1-hour prior to OTA. Relative expression levels of indicated proteins in OTA-treated samples (normalized to ERK1/2) are shown with their ratios to the corresponding control group.

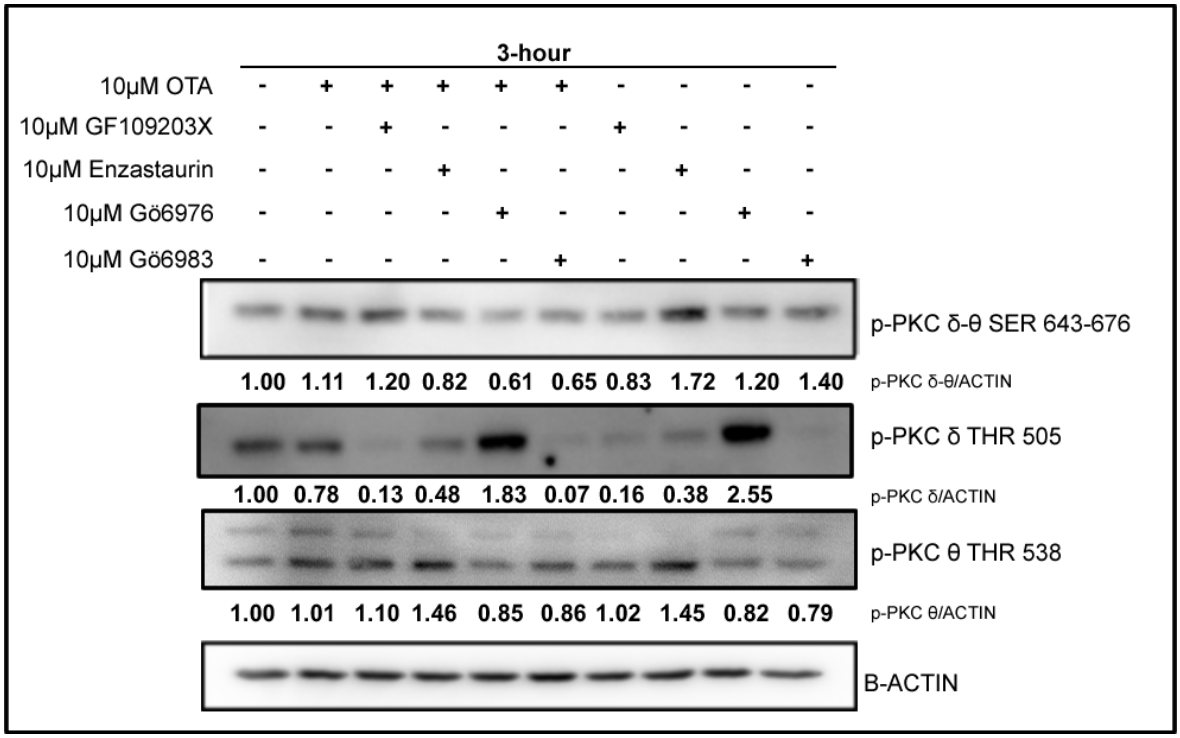

Figure 5.10. Effects of PKC inhibitors on novel PKC isoform activity induced by OTA. Cells were treated either with 10μM OTA alone or in combination with10μM GF109203X, 10μM Enzastaurin, 10μM Gö6976, and 10μM Gö6983 respectively for indicated time points. 0.1% EtOH and DMSO were used as vehicles. Inhibitors were given to HK-2 cells 1-hour prior to OTA. Relative expression levels of indicated proteins in OTA-treated samples (normalized to actin) are shown with their ratios to the corresponding control group.

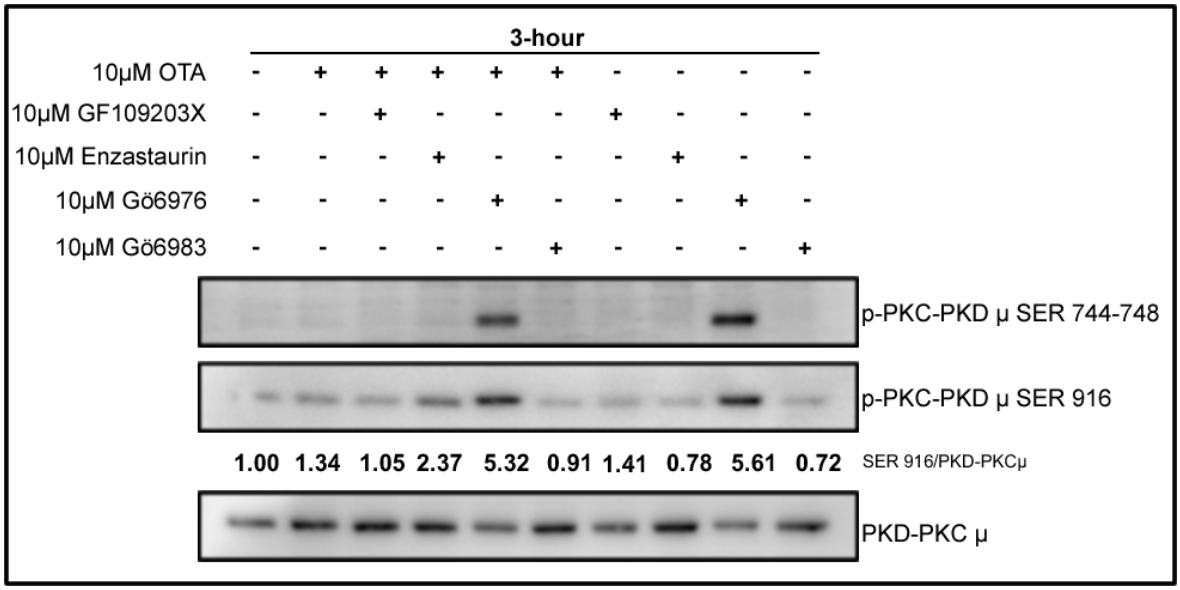

Figure 5.11. Effects of PKC inhibitors on novel atypical PKC isoform activity induced by OTA. Cells were treated either with 10μM OTA alone or in combination with10μM GF109203X, 10μM Enzastaurin, 10μM Gö6976, and 10μM Gö6983 respectively for indicated time points. 0.1% EtOH and DMSO were used as vehicles. Inhibitors were given to HK-2 cells 1-hour prior to OTA. Relative expression levels of indicated proteins in OTA-treated samples (normalized to PKD-PKCμ) are shown with their ratios to the corresponding control group.

5.6. Effect of Cholera Toxin (CTX) and Pertussis Toxin (PTX) on activation on ERK1/2

Based on proteomics analysis performed in our laboratory, Cholera Toxin (CTX) and Pertussis Toxin (PTX) were utilized in order to investigate the relationship between ERK1/2 activation and GPCR signaling. CTX is a complex released by a bacterium called *Vibrio cholerae*. CTX is termed as a toxic substance because this complex can cause severe damage in intestines resulting in diarrhea. In cells exposed to this complex, CTX catalyses ADP-ribosylation of the $G_{\alpha s}$ subunit of heterotrimeric G proteins and subsequently causes $G_{\alpha s}$ to be remained active. Then the adenylate cyclase enzyme is activated by $G_{\alpha s}$ protein and finally production of cyclic adenosine monophosphate (cAMP). Ultimately, cAMP level of the cell increases [109, 110].

PTX is produced by *Bordetella pertussis* bacteria causing excessive cough and fever known as whooping cough. PTX is released from this highly contagious bacterium. This toxin has also been associated with the GPCR signal transduction. When

cells are exposed to PTX, this toxin causes inhibition of $G_{\alpha i}$ subunit of G proteins via ADP-ribosylation. $G_{\alpha i}$ subunit has an inhibitory role for adenylate cyclase enzyme in the cell. Therefore, the inhibition of this subunit give rise to adenylate cyclase activation and an increase in the cAMP levels in cells [111, 112].

cAMP acts as a second messenger in the cell and has vital roles in many cellular processes. Adenylate cyclase is responsible for cAMP synthesis [113]. Adenylyl cyclase is an enzyme catalyzing cAMP production by converting adenosine triphosphate (ATP) to 3', 5'-cyclic AMP and pyrophosphate [114]. Adenylate cyclase normally found anchored in the plasma membrane. Its activation occurs in G-protein dependent manner. GPCRs anchored with $G_{\alpha s}$ proteins are responsible for the activation of adenylate cyclase. cAMP is responsible for conveying signaling messages via cAMP-dependent pathway. When adenylate cyclase is activated, it catalyzes multiple cAMP synthesis and intracellular cAMP increases subsequently. cAMP activates cAMP-dependent kinase PKA, and PKA regulates further cellular process either by entering nucleus or by phosphorylating downstream proteins [115]. cAMP levels can be decreased via decomposition into AMP by phosphodiesterases [116]. Additionally, $G/_{o}$-protein coupled receptors can cause decrease in intracellular cAMP levels by inhibiting adenylate cyclase [117]. It is well accepted that these two toxins have roles in the alteration of G protein signalization by following different paths.

HK-2 cells were treated with these toxins and ERK1/2 activation change was observed. In order to determine treatment concentrations of these toxins, several studies were performed beforehand. Activation change of ERK1/2 protein was analyzed via Western blotting after HK-2 cells treated with CTX and PTX chemicals individually or in combination with OTA. When cells were exposed to OTA alone, ERK1/2 activation increased from the early hours of treatment as expected. When cells were treated with CTX in addition to OTA, however, OTA-mediated ERK1/2 activation fell especially in third and sixth hours of experiment. In samples treated only with CTX, a decrease in ERK1/2 activation was observed in all hours of treatment. (Figure 5.12).

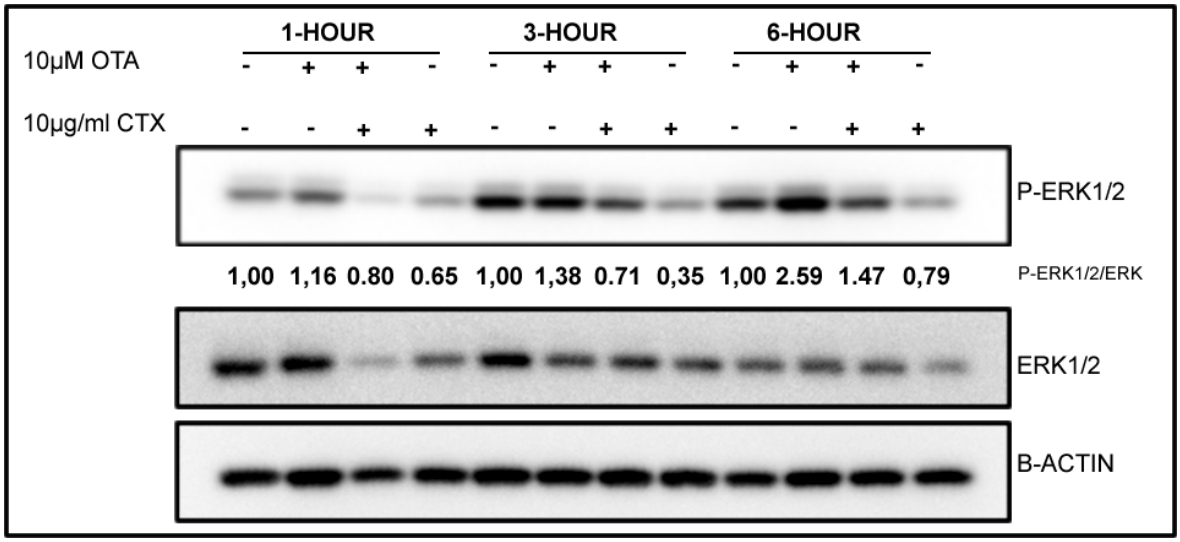

Figure 5.12. Effect of CTX treatment on the activation of ERK1/2 in HK-2 cells. Cells were treated either with 10μMOTA, 10μg/ml CTX, or 0.1% EtOH as vehicle for indicated time points. CTX treatment started 4 hours prior to OTA treatment. ERK1/2 phosphorylation was normalized to that of ERK1/2 and presented as fold change, relative to the corresponding control group.

OTA demonstrated its activation effect on ERK1/2 protein in HK-2 cell line at third hour of treatment. When cells were exposed to OTA with PTX, ERK1/2 activation diminished compared with that of cells treated with OTA alone. There was also a reduction in basal ERK1/2 phosphorylation in cells that had been treated only with PTX compared with treated with vehicle alone. (Figure 5.13).

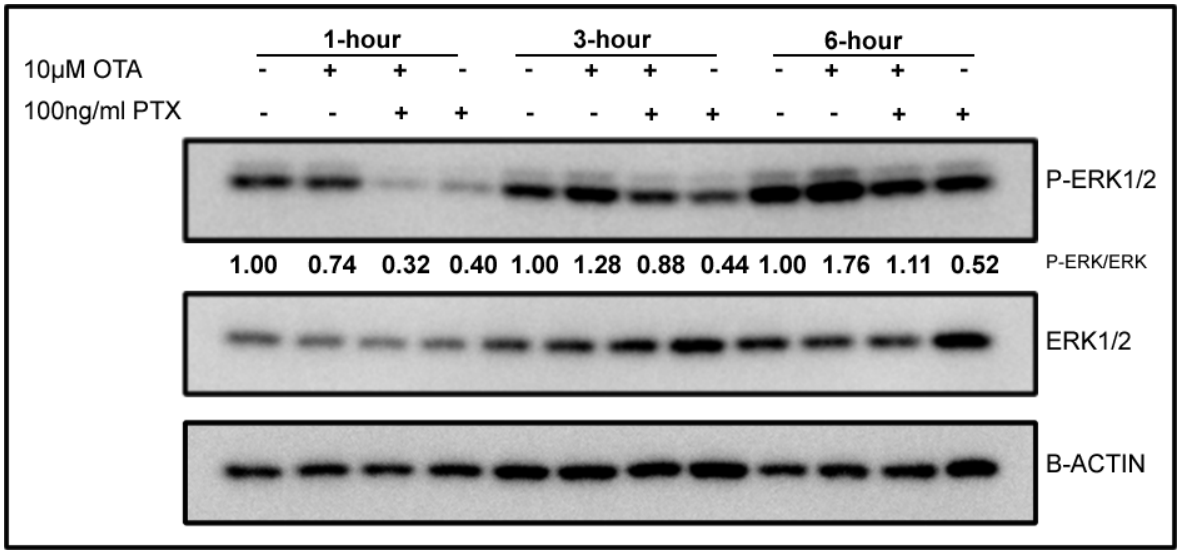

Figure 5.13. Effect of PTX treatment on the activation of ERK1/2 in HK-2 cells. Cells were treated either with 10μMOTA, 100ng/ml PTX, or 0.1% EtOH for indicated time points. PTX treatment occurred 4 hours prior to OTA treatment. ERK1/2 phosphorylation was normalized to that of ERK1/2 and presented as fold change, relative to the corresponding control group.

5.7. Alteration in activation of ERK1/2 in the presence of high cAMP level in the cell.

Both CTX and PTX are expected to increase intracellular levels of cAMP. They accomplish this role by modulating different G_α subunits of heterotimeric G protein complexes on the cell membrane. Since there was a significant reduction in the OTA-induced phosphorylation level of ERK1/2 protein when co-treated with these reagents, it was hypothesized that OTA somehow interacts with a GPCR and then decreases the level of cAMP from the early hours of treatment. Then, low intracellular levels of cAMP trigger downstream signaling pathways and finally ERK1/2 activation in HK-2 cells. In order to test our hypothesis, HK-2 cells were treated with chemicals known to have regulatory role in intracellular levels of cAMP. After treating HK-2 cells with these chemicals in the presence or absence of OTA, ERK1/2 activation changes were observed.

5.7.1. The effect of 8-bromoadenosine 3', 5'-cyclic monophosphate on the phosphorylation levels of ERK1/2

8-bromoadenosine 3', 5'-cyclic monophosphate (8-bromo) is a cell-permeable cAMP analog and it shows higher resistance to hydrolysis by phosphodiesterases than cAMP [118]. It was used in order to mimic increased intracellular cAMP levels and observe changes in ERK1/2 activation.

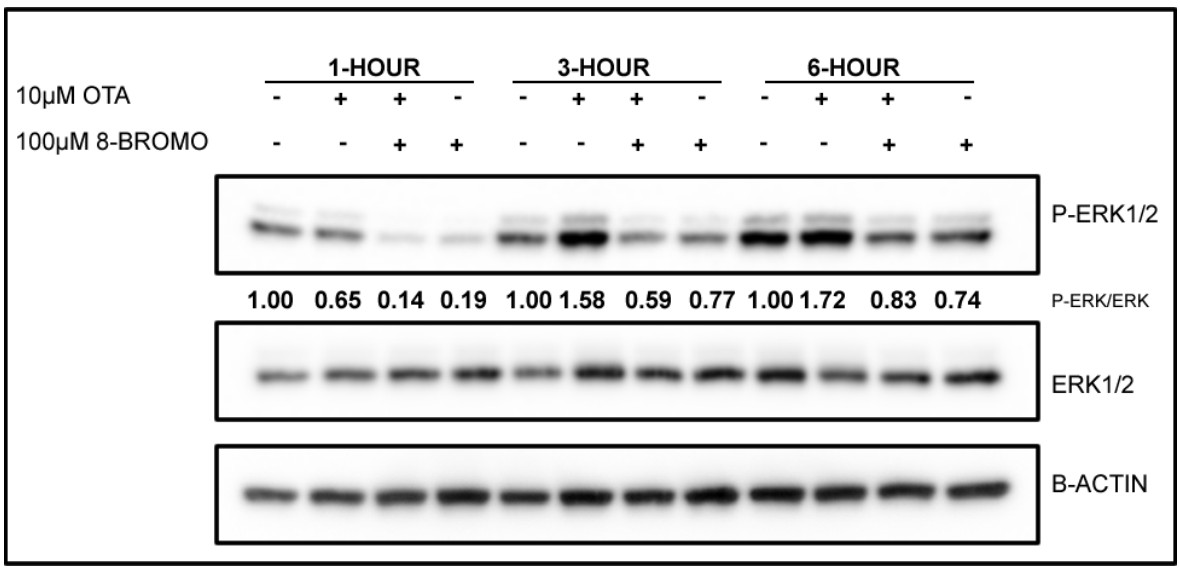

Figure 5.14. Effect of 8-bromo treatment on ERK1/2 activation in HK-2 cells. Cells were treated either with 10μM OTA, 100μM 8-bromo, or 0.1% EtOH and 0.1%N ammonium hydroxide as vehicle for indicated time points. 8-bromo treatment occurred 1 hour prior to OTA treatment. ERK1/2 phosphorylation was normalized to that of ERK1/2 and presented as fold change, relative to the corresponding control group.

Cell groups treated just with OTA exhibited high levels of phosphorylation in ERK1/2 at third and sixth hours of treatment. ERK1/2 activation dramatically decreased in cells treated either with 8-bromo alone or in combination with OTA at first hour of treatment. 8-bromo maintained regression of OTA-induced ERK1/2 phosphorylation in third and sixth hours of experiment suggesting that the low levels of cAMP caused by OTA treatment leads to ERK1/2 activation (Figure 5.14).

5.7.2. The effect of Forskolin on the phosphorylation levels of ERK1/2

Forskolin has the ability of activating adenylate cyclase by a mechanism that is independent from GPCRs on the membrane. As a result of forskolin treatment, intracellular levels of cAMP increase [119]. HK-2 cells displayed high levels of phosphorylation of ERK1/2 protein when they were treated with OTA only at third and sixth hours of experiment. With the addition of Forskolin to OTA treatment, ERK1/2 activation dramatically decreased. Forskolin also had a basal effect on HK-2 cells where it caused ERK1/2 inhibition. Forskolin demonstrated its inhibitory role on ERK1/2 activation at all time-points of OTA treatment (Figure 5.15).

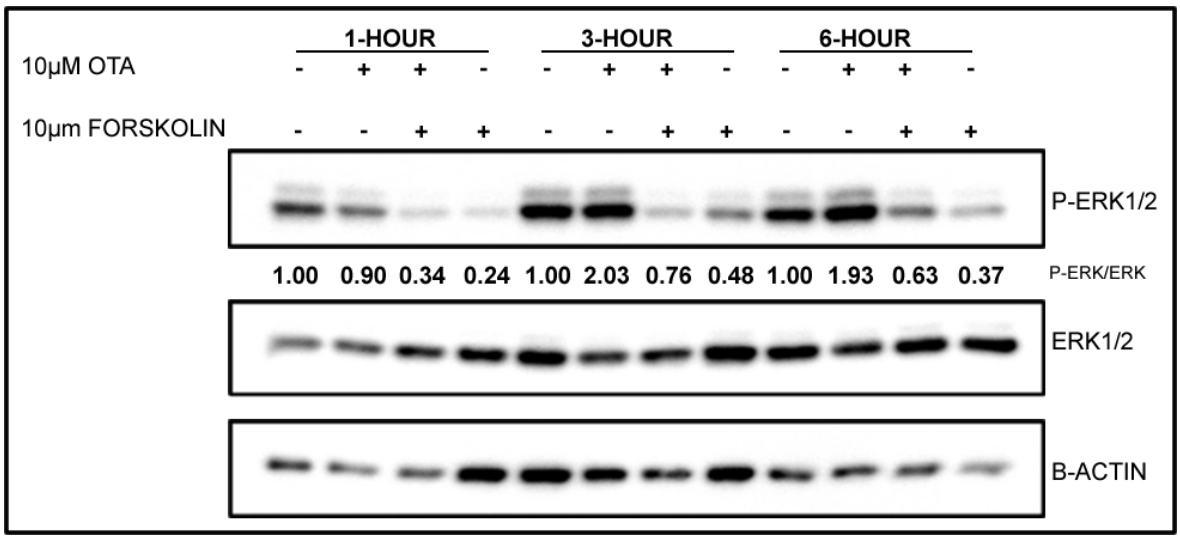

Figure 5.15. Effect of Forskolin treatment on ERK1/2 activation in HK-2 cells. Cells were treated either with 10 μM OTA and 10μM Forskolin or 0.1% EtOH and DMSO as vehicle for indicated time points. Forskolin treatment was started 1 hour prior to OTA treatment. ERK1/2 phosphorylation was normalized to that of ERK1/2 and presented as fold change, relative to the corresponding control group.

5.7.3. The effect of 3-isobutyl-1-methlyxanthine on the phosphorylation levels of ERK1/2

3-isobutyl-1-methlyxanthine (IBMX) is a cAMP and cGMP phosphodiesterase inhibitor. When these enzymes are inhibited, intracellular cAMP levels increase [120]. OTA displayed its activation role on ERK1/2 in third and sixth hours of treatment. However, IBMX did not reduce on OTA-induced ERK1/2 activation. Cell groups treated both with OTA and IBMX revealed higher ERK1/2 activation than groups treated with OTA alone at third and sixth hours of treatment (Figure 5.16).

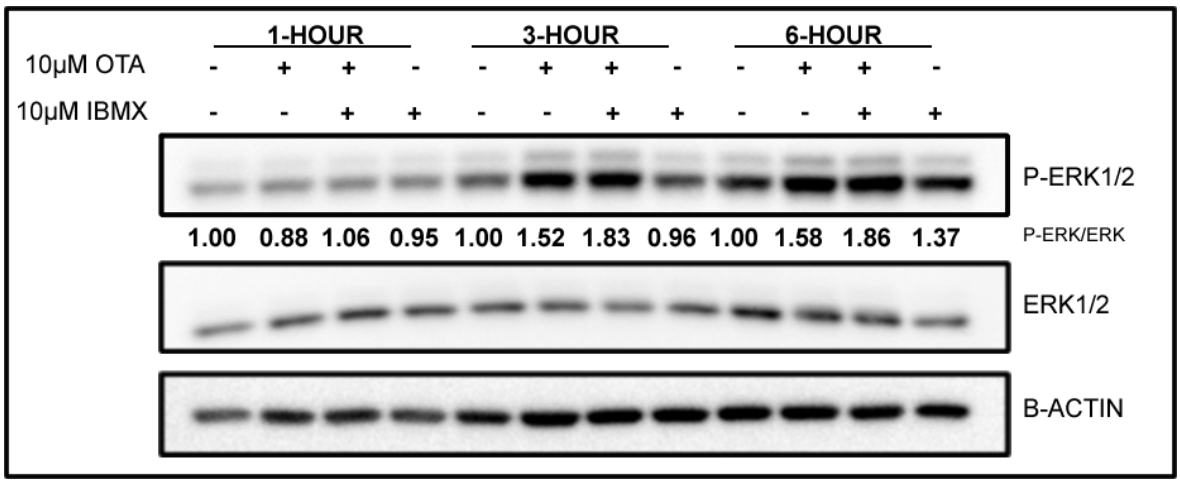

Figure 5.16. Effect of IBMX treatment on ERK1/2 activation in HK-2 cells. Cells were treated either with 10μM OTA, 10μM IBMX, or 0.1% vehicle (EtOH and DMSO) for indicated time points. IBMX treatment was started 1 hour prior to OTA treatment. ERK1/2 phosphorylation was normalized to that of ERK1/2 and presented as fold change, relative to the corresponding control group.

5.7.4. The effect of isoproterenol hydrochloride on the phosphorylation levels of ERK1/2

Isoproterenol hydrochloride (ISO) has been known to be an agonist of β-adrenergic receptor. β-adrenergic receptors activate adenylate cyclase through action of G proteins when bound by its ligands. Therefore, intracellular cAMP levels increase when ISO binds to β-adrenergic receptor [121]. compared with the control groups, cells treated only with OTA displayed increased activity of ERK1/2 protein in third and sixth hour of treatment. It was observed that OTA-induced ERK1/2 activation diminished dramatically in the presence of ISO at third hour of experiment. There was an activation

increase in cells exposed to OTA at sixth hour of experiment as expected, however; ISO did not reduce OTA-induced ERK1/2 activation at sixth hour of experiment (Figure 5.17). Nevertheless, these experiments have to be repeated to confirm the results.

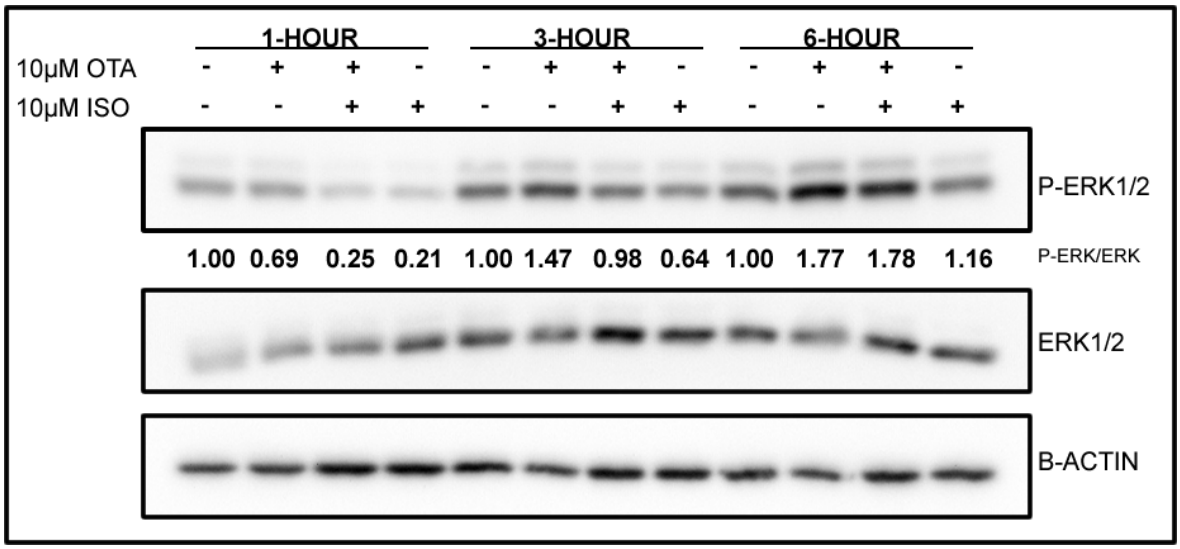

Figure 5.17. Effect of ISO treatment on ERK1/2 activation in HK-2 cells. Cells were treated either with 10μM OTA, 10μM ISO, or 0.1% EtOH as vehicle for indicated time points. ISO treatment was started 1 hour prior to OTA treatment. ERK1/2 phosphorylation was normalized to that of ERK1/2 and presented as fold change, relative to the corresponding control group.

5.7.5. The effect of N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89) on the phosphorylation levels of ERK1/2

So far in this study, it was revealed that increases in the intracellular cAMP levels diminish OTA-induced ERK1/2 activation in HK-2 cells via Western blot analyses. However, it was unclear whether cAMP-dependent inhibition of ERK1/2 occurs through canonical cAMP signaling pathway involving cAMP-dependent Protein Kinase (PKA). In order to illuminate this part of signaling further, downstream target of cAMP was investigated in relation to ERK1/2 activation. Changes in ERK1/2 phosphorylation was investigated in the presence of PKA inhibitor H-89 in combination with OTA [122]. Cells exposed to OTA alone display higher levels of ERK1/2 phosphorylation compared with control groups for the third and sixth hours of exposure. When experimental groups were treated with H-89 in addition to OTA, phosphorylation of ERK1/2 protein increased compared not only to control groups but also to

OTA-treated samples. Activation increment was observed to begin at first hour of treatment and it was maintained during the third and sixth hours of treatment (Figure 5.18).

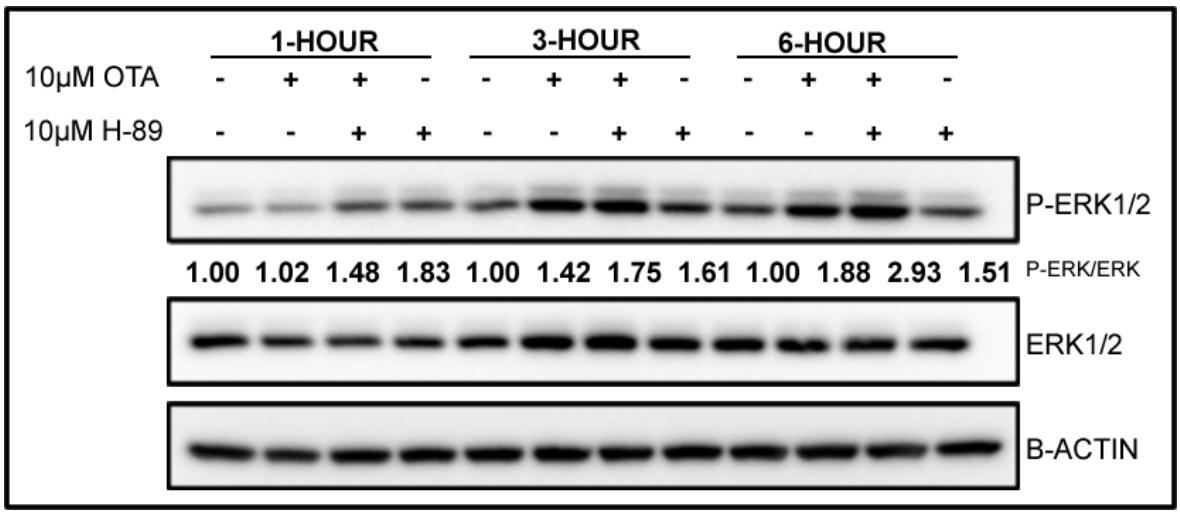

Figure 5.18. Effect of H-89 treatment on ERK1/2 activation in HK-2 cells. Cells were treated either with 10μM OTA, 10μM H-89, or 0.1% vehicle (EtOH and DMSO) for indicated time points. H-89 treatment started 1 hour prior to OTA treatment. ERK1/2 phosphorylation was normalized to that of ERK1/2 and presented as fold change, relative to the corresponding control group.

5.8. Effect of OTA treatment on intracellular cAMP level

In order to observe the direct effect of OTA exposure on intracellular cAMP levels, HK-2 cells were treated with OTA and analyzed by cAMP chemiluminescent assay. As indicated in methods part, this assay depends on the competition between cAMP that comes from the test sample and fixed amount of HRP-linked cAMP. They compete for binding to immobilized anti-cAMP antibody in each well. Consequently, the more cAMP in the sample, the less signal from HRP is observed. In OTA-treated HK-2 cells, the luminescence signal was slightly higher than control group at first hour of experiment suggesting that cAMP levels decreased when cells were treated with OTA. At third hour of treatment, cAMP levels in OTA-exposed cells were lower than that of control group. cAMP increasing agents were used for further confirmation of our results. They could negate the effect of OTA on cAMP level at third hour of treatment. In cells treated only with cAMP agents, forskolin could increase basal cAMP level also in HK-2 cells. At sixth hour of treatment, there was no significant difference in cAMP

levels between OTA-treated and control group. All in all, intracellular levels of cAMP decreases in OTA-treated cells compared with non-treated cell groups at the first and third hours of treatment (Figure 5.19).

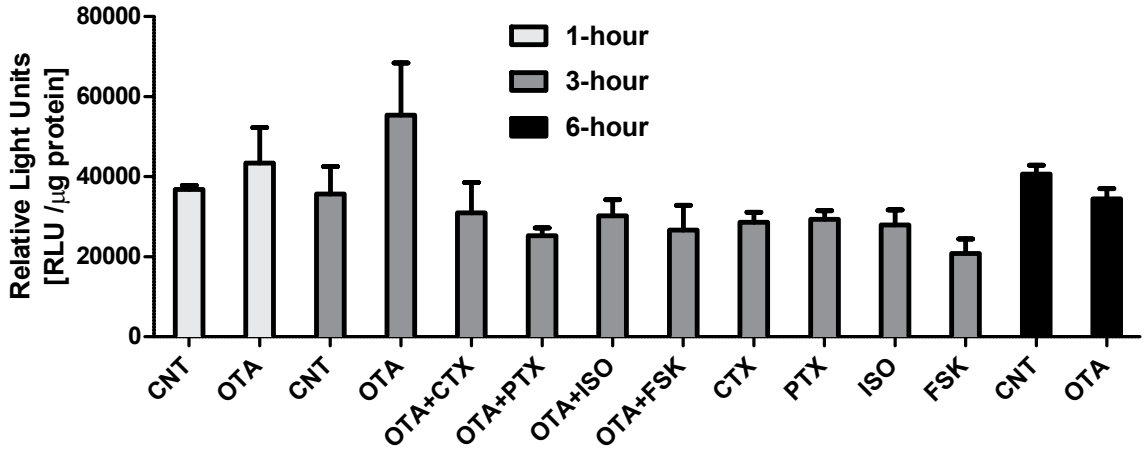

Figure 5.19. Effect of OTA alone or in combination with CTX, PTX, ISO, IBMX, and Forskolin on cAMP levels in HK-2 cells. Cells were treated with 10μM OTA, 10μg/ml CTX, 100ng/ml PTX, 10μM ISO, and Forskolin. 0.1% EtOH and DMSO were used as vehicle. CTX, PTX, ISO, and Forskolin treatment started 1 hour prior to OTA treatment.

5.9. Effect of intracellular cAMP increase on OTA-induced apoptotic cell death

Thus far our results show that increase in cAMP levels causes ERK1/2 inhibition in HK-2 cells. We used chemicals affecting intracellular cAMP levels through either G protein-dependent or -independent mechanisms. Their common feature is increasing intracellular cAMP level and they reduce OTA-induced ERK1/2 activation. In addition, when cAMP-dependent kinase PKA was inhibited by H-89, OTA-induced ERK1/2 activation raised more. Moreover, it was previously shown that OTA causes sustained MEK/ERK1/2 activation and this activation induces apoptosis in HK-2 renal proximal tubule epithelial cell line. In order to investigate whether cAMP levels are involved in OTA-induced apoptotic cell death, caspase 3/7 activity assay was performed. Even though OTA was shown to induce apoptosis at 24 hours of OTA treatment, it was not known whether this apoptotic cell death starts earlier or not. At sixth hour of OTA exposure, caspase 3/7 activities increased moderately compared with control group. ISO, IBMX and Forskolin did not reduce apoptotic effect of OTA. Furthermore, they

increased caspase 3/7 activity when they are given to HK-2 cells together with OTA. On the other hand, H-89 also increased caspase 3/7 activity in HK-2 cells. At 24^{th} hour of experiment, caspase 3/7 activity was increased in OTA-treated cells. However, cAMP intensifiers also caused increase in the caspase activity (Figure 5.20).

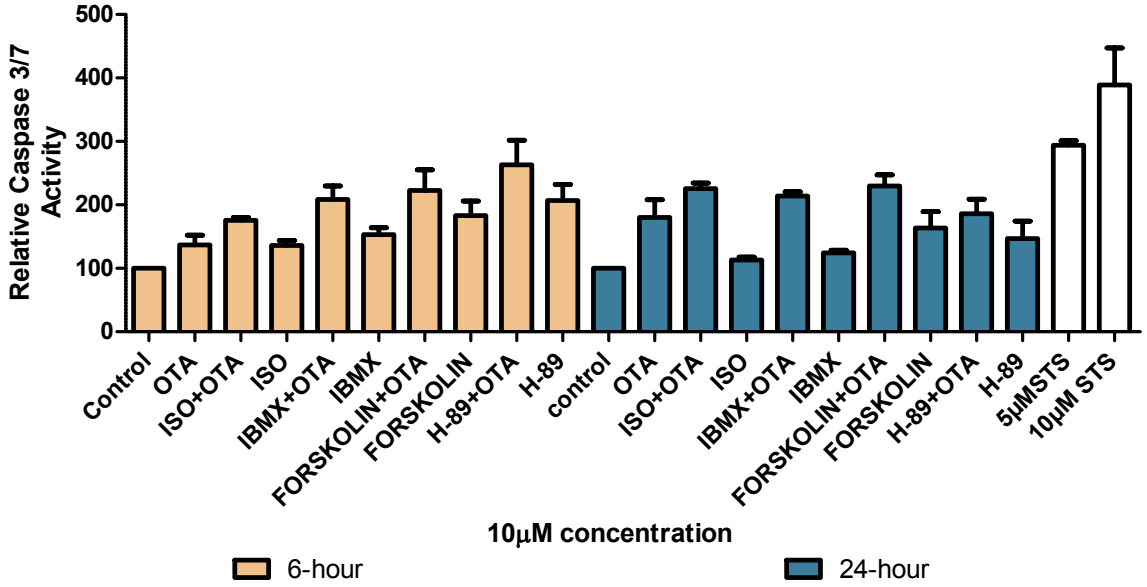

Figure 5.20. Caspase 3/7 Glo activity assay. Cells were treated with 10μM OTA or 10μM ISO, IBMX, Forskolin and H-89 respectively. 0.1% EtOH and DMSO were used as vehicles. Staurosporine (STS) was used as positive control for Caspase 3/7 Glo assay.

6. DISCUSSION

OTA is widely accepted as a nephrotoxic mycotoxin due to having various toxicopathological effects on animal kidneys [123]. Although OTA is frequently found in food as a contaminant, its nephrotoxic and carcinogenic effects in humans is poorly understood.

In our previous study, we demonstrated that OTA induces sustained MAPK/ERK1/2 activation in HK-2 cell line [32]. However, the upstream effector of ERK1/2 activation remained obscure. In this thesis, a number of candidate upstream activators were screened by chemical inhibitors of respective proteins throughout the study in order to elucidate the upstream effector(s) of OTA-induced ERK1/2 activation (Figure 1.12).

Integrins are important cell membrane proteins playing role in transmission of vital signaling processes to intracellular compartments of the cell. They have been implicated to have crucial roles in cell development and certain pathological processes. Integrins do not have any enzymatic activity, recruitment of cytosolic kinases such as ILK maintain the transmission of the signal. ILK was shown to be involved in the phosphorylation of ERK1/2, p38 MAPK, JNK, and PKB in hepatic stellate cells [124]. In leukemic and bone marrow stromal cells, inhibition of ILK with a specific inhibitor resulted in suppression of ERK1/2 activity [125]. Upregulation of ILK expression resulted in elevation of ERK1/2 phosphorylation in mammary gland of mice [53]. In addition to this studies, our previous data showing expression level regulation under OTA exposure made us think that ILK might be an upstream activator for OTA-induced ERK1/2 activation in HK-2 cells. The expression of the most known variants of integrins $\alpha 5$, $\beta 1$, and $\beta 3$ increased at the beginning of OTA treatment. ILK expression change was valuable to observe since integrins show their signaling effect through ILK proteins. ILK protein expression was observed to increase at the first hours of OTA treatment similar to integrin $\beta 1$ and $\beta 3$. The expression pattern of ILK is in concordance with that of integrins as expected because ILK interacts with β chain of integrin heterodimers (Figure 5.2).

Our findings are insufficient to conclude that ILK is an upstream effector of ERK1/2 activation. Further experiments should be performed to validate ILK involvement in OTA-induced ERK1/2 pathway in HK-2 cell line. ILK may be acting downstream of integrin receptors but we might not be able to see it since we examined the endogenous protein levels of total ILK. However, it is known that, ILK autophoshorylation as a post-translational modification is crucial for its activation [54]. Besides, ILK was shown to be phosphorylated directly in ILK-mediated signaling [126]. Observation of phosphorylated form of ILK under OTA treatment should be tested for further testing the hypothesis. Additionally, ILK can be inhibited chemically and/or genetically in HK-2 cells and subsequently changes in OTA-induced ERK1/2 activation can be examined.

FAK was another candidate for being an upstream effector for ERK1/2 activation. FAK enzymes are known to be regulated by integrin clustering. Right after integrin clustering and FAK recruitment, autophoshorylation of FAK on Y397 residue takes place. Phosphorylated Y397 residue forms the binding site for Src family proteins, PI3K and PLC γ enzymes. Recruitment of additional enzymes at the autophosphorylation site leads to phosphorylation at catalytic domain (Y576/577) and carboxy-terminal region (Y925) [127]. FAK was chosen as a candidate based on an ample number of *in vivo* and in vitro experiments that show FAK as an upstream effector of MAPK/ERK1/2 pathway [66, 67]. Transfection of pancreatic cancer cells with FAK siRNA or treatment with anti-β1 integrin antibody inhibited the Ras activation and subsequently ERK1/2 phosphorylation [128]. In a study performed in rats, increases in diastolic pressure resulted in high level of FAK phosphorylation leading to activation of ERK1/2 and they were observed as localized in cardiac myocytes [129].

However, there was no discernable activation in FAK residues under OTA treatment. Only in the first hour of experiment, a slight increase in phosphorylation at Y576/77 and Y925 residues was observed (Figure 5.4). It was unexpected to see activation at Y576/77 and Y925 residues whereas there was no activation at Y397 residue. Generally, autophosphorylation works as an 'on'-'off' center of kinases. On the other hand, phosphorylation can easily be reverted by phosphatases. We might not catch the

Y397 phosphorylation because of a timing problem associated with the experimental set-up used in this study. Activation of FAK could be very transient so that the activation of FAK could not be observed. Therefore, earlier time point should be examined. There are various studies showing FAK activation at earlier time points [130–132]. There are also other studies claiming that OTA reduces FAK phoshorylation in some cell types [69]. The reason for observing opposing results in regulation of FAK residues can arise from experimental set-up, treatment conditions and cell type differences. In an *in vitro* study performed with kidney proximal tubular cells, it was shown that FAK phosphorylation was reduced under 10μM OTA treatment [133]. Since we observed reduction in FAK phosphorylation at later time points of OTA, our result seems to corroborate with other studies. Nevertheless, further experimentation is required in order to validate involvement of FAK with ERK1/2 activation.

Possible involvement of PKC family was also investigated as a candidate in OTA-induced ERK1/2 activation. PKC has many isoforms and they have been divided into three groups as conventional, novel and atypical forms [134]. Almost all the isoforms of PKC were examined in order to assess whether they are involved in OTA-induced ERK1/2 activation. In agreement with our previous results, ERK1/2 phosphorylation was increased at three hours of OTA treatment. Contrary to our anticipation, OTA-induced ERK1/2 activation was further enhanced by the addition of GF109203X and Enzastaurin. These two chemicals might interfere with ERK1/2 activity and regulate ERK1/2 phosphorylation via different way. If PKC activates ERK1/2 in OTA-exposed HK-2 cells, (α and β) isoforms that are inhibited by GF109203X and Enzastaurin might not be responsible for the activation. On the other hand, the use of Gö6976 and Gö6983 in combination with OTA diminished OTA-induced ERK1/2 activation. This observation suggests that PKC PKCμ, PKCδ might work as upstream activators in the cell, therefore; their inhibition results in reduction of ERK phosphorylation in OTA treated HK-2 cells. In accordance with our observations, it was reported that OTA increased the phosphorylation of atypical-PKC and downstream activation of ERK1/2 in rat kidney [79].

Nevertheless, inhibiting PKC enzyme by using chemicals will not provide enough support for the hypothesis that PKC is an upstream effector for ERK1/2 activation under OTA exposure. However, the use of these chemicals is helping us to focus in one or more specific PKC isoform for a closer examination. Candidate isoforms can be genetically inactivated and then ERK1/2 activation can be examined in more detail. The novel (δ) and novel atypical (μ) isoforms can be further investigated in terms of being at the upstream position of MAPK/ERK1/2 signaling activation by OTA. There are number of studies showing that these three isoforms can act as upstream activators of ERK1/2 in various cell types (Table 6.1)

Table 6.1. Studies showing three PKC isoforms as an upstream activator for ERK1/2

Experiment	**Cell Type**	**Result**
PKCμ overexpression	Swiss3T3	Overexpression of PKCμ results in prolonged activation of the MAPK/ERK1/2 pathway [135].
Knockdown of endogenous PKCμ	LNCaP	Down regulation of endogenous PKCμ decreases ERK1/2 activation in LNCaP cells [136].
Co-expression of constitutively active PKCη with PKCμ	HEK293	PKCη directly phosphorylates PKCμ and subsequently ERK1/2 is activated [137].
Knockdown of PKCμ in prostate cancer cells	PC3 and DU145	PKCμ inhibition causes reduction in ERK1/2 activation [138]
6-OHDA treatment to induce toxicity	PC12	Protein kinase Cδ-mediated cell death occurs via activation of ERK1/2 [139].
Activation of PKCδ via point mutation	NIH3T3 and COS1	PKCδ activates ERK1/2 [76].

GPCRs were the last candidates that have the highest potential for being an upstream effector of MAPK/ERK1/2 signaling under OTA exposure. This is mainly because of the fact that the proteins that showed an increased expression pattern in OTA-treated HK-2 cells included members of G-protein related signaling pathway according to our unpublished proteomics data (Figure 1.5).

As mentioned before, CTX and PTX are commonly used in experiments investigating G protein-dependent signaling. Both of them ultimately causes increase in the intracellular cAMP levels [109, 112]. Decrease in OTA-mediated ERK1/2 activation after CTX and PTX treatment was very noticeable. Among all upstream effector candidates, HK-2 cells displayed the most apparent response to the modulation of G-protein dependent signaling. Suppression of OTA-induced ERK1/2 activity in the presence of CTX and PTX was worth studying further.

In order to test whether reduced ERK1/2 activation occurred as a consequence of increased level of intracellular cAMP, 8-bromo, a cAMP analog was given to HK-2 cells in combination with OTA. It was clearly demonstrated that artificially increased cellular cAMP levels reduced the OTA-induced ERK1/2 activation and also diminished basal level of phosphorylated ERK1/2 in HK-2 cells.

Then, we wanted to observe if our results were supported when intracellular cAMP levels increased by means of different principles in the cell. Forskolin and IBMX are two chemicals activating adenylate cyclase and inhibiting cAMP phosphodiesterases, respectively. They both increase intracellular cAMP level [119, 120]. At all time points of treatment, Forskolin in combination with OTA caused reduction in ERK1/2 phosphorylation in HK-2 cells. These results suggest that chemically activated adenylate cyclase increases intracellular level of cAMP and subsequently causes diminished ERK1/2 activation confirming our previous results. As for the IBMX co-treatment, it was expected to increase cAMP levels in the cell and negate the MAPK/ERK1/2 activating effect of OTA. Contrary to our expectations, IBMX did not repress the OTA-induced ERK1/2 activation at any hours of treatment. In addition, ISO was used to see whether increase in cAMP through activation of G proteins would also cause reduction in OTA-induced ERK1/2 activation as in CTX. As expected, ISO caused reduction in OTA-induced ERK1/2 activation. However, it could not maintain this reduction beyond third hour of experiment. Its reducing effect on p-ERK1/2 was apparent only in first and third hours of experiments.

To elucidate more whether inhibition of ERK1/2 phosphorylation occurs via cAMP-dependent signaling pathway, a PKA inhibitor (H-89) was given to HK-2 cells in combination with OTA [122]. H-89 enhanced the OTA-induced ERK1/2 activation. It supports the idea that cAMP dependent pathway can be responsible for the ERK1/2 inhibition in the cells under OTA exposure.

In order to understand whether OTA causes decrease in cAMP level, ELISA assay was performed. It was detected that OTA reduced the intracellular level of cAMP. Agents which are used to increase cAMP level caused an increase in cAMP levels in HK-2 cells as expected (Figure 5.19). Even so, it is not certain that OTA causes cAMP decrease in the cell since this assay was performed only once. In order to validate OTA-mediated cAMP regulation, the assay should be repeated.

In our previous work, it was suggested that OTA-induced apoptosis takes place through ERK1/2 activation [32]. Therefore, we wanted to test whether reduction in ERK1/2 activity via cAMP elevating agents might cause a decrease in caspase 3/7 activity. We observed increased caspase 3/7 activation in OTA-treated cells at 24^{th} as expected. Previously the caspase activities had not been examined at earlier time points of OTA exposure. In this study, it was shown that OTA increases caspase 3/7 activation even at sixth hour, albeit in a moderate level. However, agents that elevate cAMP levels did not reduce OTA-induced caspase 3/7 activity as anticipated. On the contrary, they increased caspase 3/7 activities when they were applied to the cells with or without OTA at the doses utilized in this study. Several studies have shown their apoptosis inducing characteristics in various cell types [140–142]. It was reported that ISO at 10 uM concentration induces apoptosis in cardiomyocytes through activation of PKA [140]. Moreover, forskolin was shown to increase apoptotic cell death in myeloid leukemia cells via activating Protein Phosphatase 2A (PP2A) and subsequently reducing the activity of survival pathways [141]. In HK-2 cells, almost all agents showed increased basal caspase 3/7 activity. When these agents were used in combination with OTA, caspase 3/7 activities were further enhanced for the time points examined. Therefore, it is possible that the duration and the levels of cAMP that the cells are subject to might trigger other apoptosis inducing signaling pathways

in HK-2 cells as well and it is suggested that these assays should be repeated by using lower concentrations of the same agents.

cAMP can have opposing roles in MAPK/ERK1/2 pathway regulation in various cell types [143]. OTA might induce ERK1/2 activation through reducing intracellular cAMP levels (Figure 5.19). However, no specific mechanism can be asserted from this study. OTA might induce G-protein dependent mechanism and subsequently cause decrease in the cAMP level. OTA might also skip G-protein signaling and act directly on adenylate cyclase enzyme inhibiting its activity and consequently cAMP production. OTA might also activate cAMP phosphodiesterases and cause reduction cAMP levels in the cell (Figure 6.1).

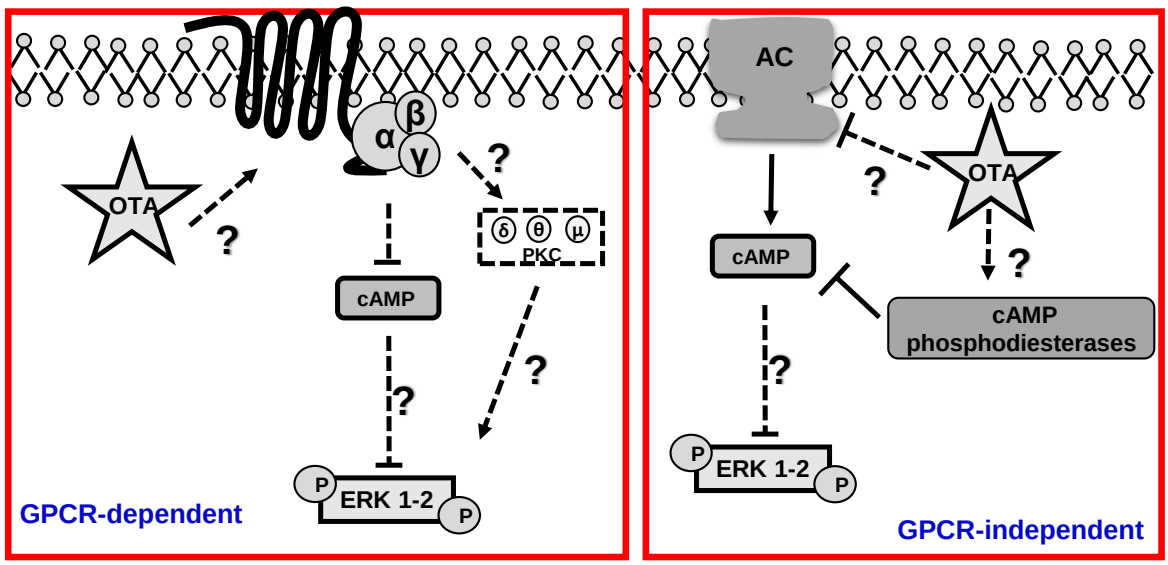

Figure 6.1. Proposed model for GPCR-dependent and independent cAMP-mediated ERK1/2 regulation in HK-2 cells

In conclusion, our results demonstrate that there is no significant increase in integrins α and β subunits. ILK, a mediator protein for integrin signaling, did not show an increased expression level, either. Moreover, there was no substantial increase in FAK phosphorylation. Nevertheless, these results do not exclude the possibility of ILK or FAK involvement in OTA-induced ERK1/2 activation in HK-2 cells. For ILK, we are still not sure if ILK and integrin get interact under OTA exposure in our cell line. Further experiments should be performed to elucidate ILK regulation under OTA exposure in HK-2 cell line. for FAK, we still do not know if FAK gets

activated earlier than our experimental set up. Since kinase activity occurs rapidly in the cell, FAK activation should be also observed at earlier time points of OTA treatment in HK-2 cells. Even though a stable PKC phosphorylation was not detected, chemical inhibition of some PKC isoforms reduced OTA-induced ERK1/2 activation. These results suggest that PKCμ, and PKCδ might be some of the possible upstream effectors in the activation of MAPK/ERK1/2 in OTA-treated HK-2 cells. Furthermore, GPCR modulation via PTX and CTX suppressed ERK1/2 activation in response to co-treatment with OTA. In addition, artificially increased intracellular cAMP caused apparent repression in ERK1/2 activation in OTA treated cells. Therefore, OTA might cause modulation of GPCR signaling resulting in a decreased level of cAMP where PKC may play an intermediary role in relaying signals from GPCR to ERK1/2. Nevertheless, further experiments are required in order to prove whether OTA stimulates GPCR signaling in HK-2 cells.

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