

## UV LIGHT STIMULATED ACTIVATION OF CAMKII IN HUMAN EPIDERMOID CARCINOMA A431 CELLS

Silvia G. Kalenderova,  
Vesela V. Lozanova,  
Maria D. Praskova,  
Valentin S. Lozanov

*Department of Medical Chemistry and  
Biochemistry,  
Medical University – Sofia,  
Bulgaria*

**Corresponding Author:**

Valentin S. Lozanov  
Department of Medical Chemistry and  
Biochemistry,  
Medical University – Sofia  
2, Zdrave Str.  
Sofia, 1431  
Bulgaria  
*email: bovalv@gmail.com*

**Received:** October 01, 2018

**Revision received:** April 25, 2018

**Accepted:** January 17, 2019

### Summary

The Ca<sup>2+</sup>/calmodulin dependent kinase II (CaMKII) represents a family of serine/threonine kinases with diverse functions, implicated in various signalling pathways in the cell, especially in those controlling cellular proliferation and cellular death. The human epidermoid carcinoma cell line A431 is an established model for the study of the cell cycle and cancer-associated signalling, in which the role of CaMKII is not so well elucidated. Therefore, the present study aimed to investigate the role of CaMKII in the induction of apoptosis in the cell line A431 following ultraviolet (UV) irradiation and to determine the downstream events with a focus on the caspase cascade. For the latter purpose, we employed a method initially developed for the simultaneous determination of the activity of 10 different caspase enzymes. Following exposure to UV light, we observed activation of the activator caspase-8 followed by activation of the effector caspase-3, triggering apoptosis in the epidermoid carcinoma cell line A431. The results supported a pivotal role of caspases and demonstrated that a protein kinase cascade involving caspases-8 and -3 is the main pathway driven in UV-induced apoptosis in the human epidermoid carcinoma cell line A431, and apoptotic stimuli triggering this pathway passed through activation of the kinase CaMKII.

**Key words:** CaMKII, apoptosis, caspases, cell line A431

### Introduction

One of the major cell death processes triggered by ultraviolet (UV) radiation is apoptosis. Apoptosis, or programmed cell death, is widely observed in different cell types and various organisms. This process has a characteristic morphological pattern of events involving chromatin condensation, membrane blebbing, and deoxyribonucleic acid (DNA) fragmentation.

It is well established that the human epidermoid carcinoma cell line A431 underwent apoptosis following exposure to UV light and the apoptotic process is controlled by both the extrinsic (death receptor-mediated) and intrinsic (mitochondrial) pathways [1]. Activation of caspases is involved in both the initiation and especially during the execution phase of the programmed cell death. Recent improvements in caspase studies have revised our knowledge concerning

the involvement of caspase cascades in apoptosis in response to various stimuli [2]. Caspases are usually present in the cells as inactive zymogens, requiring limited proteolysis to be converted into the active form. Currently, caspase enzymes are divided into two classes: activator and effector caspases. The class of activator caspases includes caspase-8, -9, and -10, while caspase-3, -6, and -7 belong to the class of effector caspases [1, 3].

The  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) family of enzymes represents a family of serine/threonine kinase family with diverse biological functions, the members of which are ubiquitously expressed. Activation of these kinases is mediated by binding of  $\text{Ca}^{2+}$  and calmodulin, results in phosphorylation of a variety of target proteins, involves in vital cellular processes such as  $\text{Ca}^{2+}$  homeostasis, cellular growth and cell death.

In mammalian cells, four separate CaMKII genes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) have been identified, and they produce a number of different CaMKII isoenzymes [4]. Activation of CaMKII is achieved by  $\text{Ca}^{2+}$ /calmodulin-dependent phosphorylation of a specific amino acid residue ( $\text{Thr}^{286}$ ), and this allows the kinase to switch from a calmodulin-dependent to autonomous activation, i.e. the enzyme is active in the absence of  $\text{Ca}^{2+}$ /calmodulin [5, 6]. The ability of CaMKII to pass from a  $\text{Ca}^{2+}$ -dependent into a  $\text{Ca}^{2+}$ -independent form is crucial for the enzyme and allows the transformation of a transient  $\text{Ca}^{2+}$  stimulus into a sustained physiological or pathological response [7].

CaMKII receives growing attention in recent years concerning apoptosis, notably in normal and transformed neuronal, cardiac and pancreatic cells as well as in cancer cells [8].

It was suggested that  $\text{Ca}^{2+}$ /calmodulin signalling might be involved in apoptotic pathways by sustained activation of JNK through the function in CaMKII [9]. It was also reported for a proapoptotic function of CaMKII in apoptosis induced by tumour necrosis factor  $\alpha$  ( $\text{TNF}\alpha$ ) and UV radiation, with both stimuli increasing the calmodulin-independent activity of the kinase [10]. Endoplasmic reticulum (ER) stress-induced apoptosis, which is associated with increased  $\text{Ca}^{2+}$  concentration in the cytosol and alternations in  $\text{Ca}^{2+}$ -dependent signalling pathways respectively, also involves the kinase CaMKII [11]. However, the role of CaMKII

in the apoptosis of skin epithelial cells and in particular of the epidermoid carcinoma cell line A431 has not been investigated previously.

The aim of the study was to investigate the role of CaMKII in the induction of programmed cell death in the cell line A431 with focus on the activation of CaMKII following exposure to UV light and the downstream cellular event involving the caspase enzymes.

## **Materials and Methods**

### **Materials**

Human caspase-3 (apopain, EC:3.4.22.56) enzyme and gradient grade acetonitrile were purchased from Sigma-Aldrich (Germany). The caspase substrates we used with the general sequence Ac-XXXD-AMAC (where X is any amino acid, D is aspartic acid, and AMAC is 2-aminoacridone) were synthesised in our laboratory as described previously [12].

### **Cell culture**

In the present study was used the epidermoid carcinoma cell line A431 which is an established model in the study of the cell cycle and cancer-associated cell signalling pathways. We cultured the cells in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker™, Lonza) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37°C, 5%  $\text{CO}_2$  and in a humidified atmosphere. The culture was maintained as a monolayer, and when it reached approximately 50% confluence, the cells were irradiated for 1 hour with a UV lamp operating at 312 nm and a power of 15 W. All irradiation experiments were performed in triplicates using approximately  $1 \times 10^6$  cells for each replicate. Untreated A431 cells were used as a control.

### **Western blot analysis**

Western blot analysis was performed as described previously [13]. Briefly, whole cell lysates (containing 50-100  $\mu\text{g}$  protein) of irradiated or control A431 cells were obtained, resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, UK). The membranes were then probed with anti-phospho-CaMKII antibody and anti-CaMKII antibody (both from Upstate Biotechnology, Charlottesville, VA,

USA). As a control for protein loading, after membrane stripping,  $\beta$ -actin was detected with a monoclonal anti- $\beta$ -actin antibody (Sigma, St Louis, MO, USA). Primary antibodies were detected with horseradish peroxidase (HRP)-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SuperSignal reagent (Pierce, Thermo Fisher Scientific Inc., Rockford, USA).

### Caspase activity assay

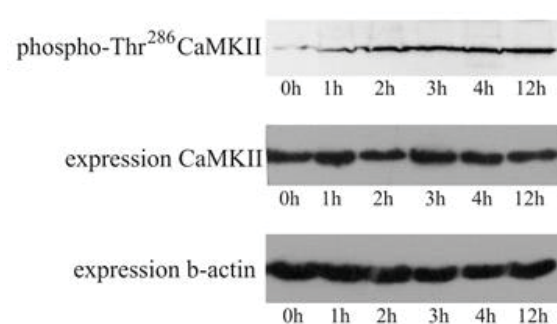
Caspase activity was determined using a set of 10 different caspase substrates using high-performance liquid chromatography (HPLC) method as described previously [14]. The enzyme reactions were carried out in caspase assay buffer (CAB) consisting of 20 mM HEPES (pH 7.4) with 2 mM EDTA, 0.1% CHAPS, 10% sucrose, and 5 mM DTT. Stock solutions of 10 mM of each substrate Ac-XXXD-AMAC were prepared in dimethyl sulfoxide (DMSO). These stock solutions were used for the preparation of a standard solution containing 250 nM of each substrate. The assay procedure was as follows: to 90  $\mu$ L of the standard solution of substrates were added 50  $\mu$ L of cell lysate. The reaction mixture was incubated for 60 min at 37°C and then acidified with 10  $\mu$ L of 0.1 M hydrochloric acid. A 10  $\mu$ L aliquot of the acidified samples was used for the subsequent HPLC analysis. The assay conditions and equipment were the same as described previously [14]. The caspase activity was measured for 6 hours after irradiation with 1-h intervals between each measurement. Samples of 12h, 24h and 36h post-irradiation were also analysed. The experiments were performed in triplicates with approximately  $1 \times 10^6$  cells used for each replicate.

## Results

### Western blot analysis

Cells from the human epidermoid carcinoma cell line A431 were irradiated with UV light with a wavelength of 312 nm for 1 h, and subsequently, the activity and protein level of the kinase CaMKII were analysed by Western blot. In the control untreated A431 cells, the basal activity of CaMKII was low and UV radiation-induced the enzyme in a concentration-dependent manner, without affecting the protein level of CaMKII. Both activity and protein level of CaMKII

were followed for a period of 24 h after the UV irradiation (Figure 1).



**Figure 1.** Western blot analysis of CaMKII in the epidermoid cell line A431 after UV irradiation for one hour

\*Samples are collected 0-12 h post-irradiation. Phospho-Thr<sup>286</sup> CaMKII refers to the quantity of the active kinase, while expression CaMKII refers to the total amount of the protein. The expression of b-actin is used as control of protein loading.

The maximal activity of CaMKII, estimated by the level of Thr<sup>286</sup> phosphorylation, was detected 3 h after the irradiation and remained stable until the end of the experiment. For the whole duration of the experiment, the protein level of CaMKII remained unchanged.

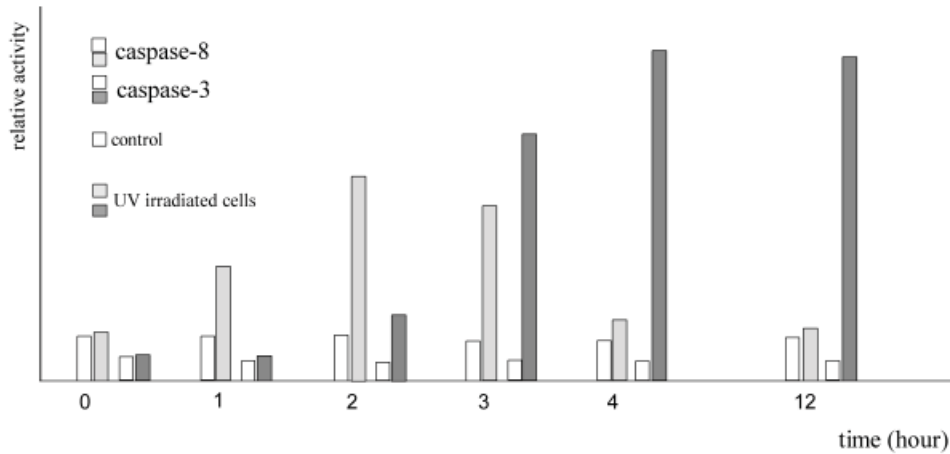
### Caspase assay

To further elucidate the role of CaMKII in UV-triggered apoptosis, we measured the activities of 10 different caspases using a previously developed original method for their simultaneous detection [14]. The enzyme activities were measured for a 6h period after irradiation with time points set at each hour. Samples at 12 h, 24 h and 36 h after irradiation have also been analysed.

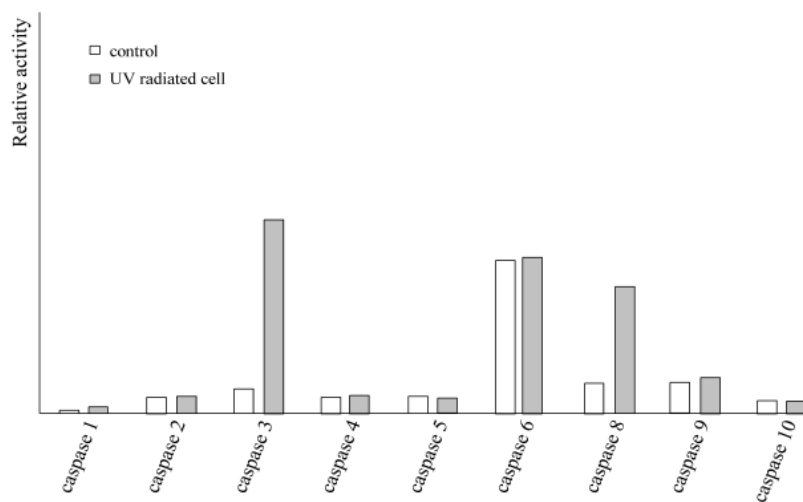
The obtained results show that UV radiation was able to induce apoptosis in the cell line A431. As early as the first hour after irradiation, there was an activation of caspase-8, this reached a peak activity on the second hour. Caspase-3 was activated at the second hour and reached maximal activity at the fourth hour, and this activity remained constant until the twelfth hour (Figure 2).

No detectable change of the activity was observed for the other tested caspases under the used experimental conditions (Figure 3).

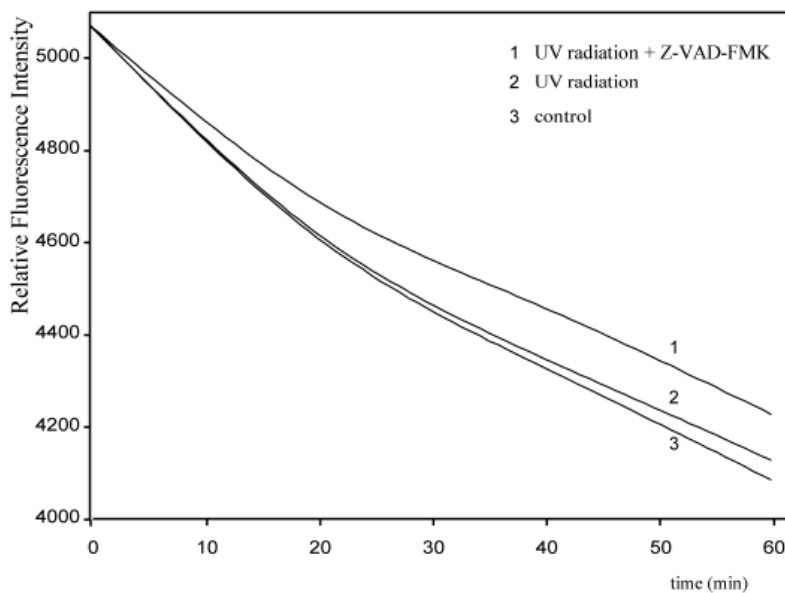
Interestingly, caspase-6 activity in both



**Figure 2.** Kinetics of activation of caspase-8 and caspase-3 after UV radiation treatment of the cell line A431



**Figure 3.** Caspase activities in UV irradiated and control A431 cells



**Figure 4.** Caspase-6 kinetic assay in the cell line A431 4 hours after UV irradiation

untreated and UV-irradiated cells was high compared to all the other tested caspase enzymes. To elucidate this finding, we further

tested the activity of caspase-6 in the presence of the inhibitor Z-VAD-FMC (Figure 4).

Z-VAD-FMC, or carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone, is a non-selective, cell-permeable inhibitor of caspase-1 and caspase-3 related proteases, which irreversibly binds to the active sites of these enzymes. In the presence of the inhibitor, the activity of caspase-6 was partially inhibited. Thus we concluded that the used substrate for caspase-6 (Ac-VEID-AMAC) is potentially a suitable substrate for other cellular enzymes and their activities prevent the proper assessment of caspase-6 activity. To our knowledge, there is no specific irreversible inhibitor for caspase-6, which would allow correct determination of the activity of caspase-6 in the human carcinoma cell line A431.

In summary, UV radiation increased the activity of the kinase CaMKII in human epidermoid tumour A431 cells leading to activation first of the activator caspase-8 and then of the effector caspase-3. Our study documents a new function for CaMKII in the transmission of signals triggering apoptosis in the human epidermoid carcinoma cell line A431.

## Discussion

UV irradiation alters a multitude of intracellular signalling pathways that participate in a variety of cellular functions. The capability of UV radiation to interact directly with the different cell membrane and cytoplasmic targets besides DNA seems to be based on the complex biologic effects it provokes [15].

Similarly, to many of the chemical and physical agents capable of inducing apoptosis, UV radiation also provokes oxidative stress through the generation of reactive oxygen species (ROS), which in turn assumes a connection between oxidative stress and apoptosis. ROS were seen as damaging for the cells; however, a growing body of evidence suggests that they are needed as activators of key cellular processes. Nowadays, ROS are considered to play a vital role in a number of cellular signalling networks employed in both physiological and pathological responses.

The calmodulin-regulated pathways appear to be the most important for the induction of apoptosis among the investigated calcium-regulated mechanisms in the cell. Calmodulin

has been implicated as both pro-survival [16] and pro-apoptotic factor [17] in the processes of programmed cell death. Since CaMKII is a major mediator of cellular  $Ca^{2+}$  effects, its inhibition is demonstrated to suppress apoptosis to a greater extent than inhibition of other  $Ca^{2+}$ -regulated enzymes such as calpain and calcineurin/PP2B [9]. The biological actions of CaMKII have been studied in detail concerning the intermediate metabolism and neural signalling [18]. Activation of CaMKII has been reported following protease activation during the apoptotic process triggered by UV irradiation and  $TNF\alpha$  [10].

Furthermore, inhibition of CaMKII blocked the induction of programmed cell death in rat hepatocytes following microcystin treatment [19]. Additionally, the apoptotic process could be induced by cadmium, and the process is dependent upon this kinase, at least in mouse mesangial cells [20]. Based on these previous reports, we focused our study on the involvement of CaMKII in the transduction of UV-triggered apoptotic signals in the epidermoid carcinoma cell line A431, and as a result, we were able to provide new insights to the signalling function of CaMKII in this cell line.

The activity of CaMKII was also found to be increased in a pro-oxidant environment, suggesting a broader function for CaMKII in cellular signalling, not the previously considered role as a mediator which links upstream oxidative stress and  $Ca^{2+}$  signals with downstream cellular responses.

## Conclusions

Based on our findings, we propose the following sequence of events triggered by UV irradiation in the human epidermoid carcinoma cell line A431. Exposure of the cells to UV radiation induces an overproduction of ROS, which subsequently increases CaMKII activity. The kinase, in turn, activates the activator caspase-8 and then the effector caspase-3 is also activated. This activation results in the induction of apoptosis and death of the cell. Apparently, the activation of CaMKII alone is not sufficient for the complete induction of apoptosis, and the activation of this kinase should be integrated with other signalling events for the complete induction and subsequent execution of apoptosis.

## Acknowledgements

This study was supported by the Council of Medical Sciences, Medical University – Sofia, under Grant No 23/2016.

## References

1. Choi KH, Hama-Inaba H, Wang B, Haginoya K, Odaka T, Yamada T, et al. UVC-induced apoptosis in human epithelial tumour A431 cells: sequence of apoptotic changes and involvement of caspase (-8 and-3) cascade. *J Radiat Res.* 2000;41(3):243-58.
2. Wu H, Che X, Zheng Q, Wu A, Pan K, Shao A, et al. Caspases: a molecular switch node in the crosstalk between autophagy and apoptosis. *Int J Biol Sci.* 2014;10(9):1072-83.
3. Shalini S, Dorstyn L, Dawar S, Kumar S. Old, new and emerging functions of caspases. *Cell Death Differ.* 2015;22(4):526-39.
4. Swulius MT, Waxham MN. Ca<sup>2+</sup>/calmodulin-dependent protein kinases. *Cell Mol Life Sci.* 2008;65(17):2637-57.
5. Ishida A, Shiger Y, Tatsu Y, Endo Y, Kameshita I, Okuno S, et al. Substrate specificity of Ca<sup>2+</sup>/calmodulin-dependent protein kinase phosphatase: kinetic studies using synthetic phosphopeptides as model substrates. *J Biochem.* 2001;129;5(1):745-53.
6. Hudmon A, Schulman H. Structure-function of the multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase II. *Biochem J.* 2002;364(3):593-611.
7. Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell.* 2008;133(3):462-74.
8. Berchtold MW, Villalobo A. The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. *Biochim Biophys Acta.* 2014;1843(2):398-435.
9. Olofsson MH, Havelka AM, Brnjic S, Shoshan MC, Linder S. Charting calcium-regulated apoptosis pathways using chemical biology: role of calmodulin kinase II. *BMC Chem Biol.* 2008;8(1):2.
10. Wright SC, Schellenberger U, Ji L, Wang H, Larrick JW. Calmodulin-dependent protein kinase II mediates signal transduction in apoptosis. *FASEB J.* 1997;11(11):843-9.
11. Timmins JM, Ozcan L, Seimon TA, Li G, Malagelada C, Backs J, et al. Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways. *J Clin Invest.* 2009;119(10):2925-41.
12. Lozanov V, Ivanov IP, Benkova B, Mitev V. Peptide substrate for caspase-3 with 2-aminoacridone as reporting group. *Amino acids.* 2009;36(3):581-6.
13. Isaeva AR, Mitev VI. Inhibition of protein kinase CK2 induces E2F1 nuclear export, formation of p21/E2F1 complexes and suppression of DNA synthesis in normal human epidermal keratinocytes. *J Dermatol Sci.* 2009;55(2):134-6.
14. Benkova B, Lozanov V, Ivanov IP, Mitev V. Evaluation of recombinant caspase specificity by competitive substrates. *Anal Biochem.* 2009;394(1):68-74.
15. López-Camarillo C, Ocampo EA, Casamichana ML, Pérez-Plasencia C, Álvarez-Sánchez E, Marchat LA. Protein kinases and transcription factors activation in response to UV-radiation of skin: implications for carcinogenesis. *Int J Mol Sci.* 2011;13(1):142-72.
16. Mishra S, Mishra JP, Gee K, McManus DC, LaCasse EC, Kumar A. Distinct role of calmodulin and calmodulin-dependent protein kinase-II in lipopolysaccharide and tumour necrosis factor-alpha-mediated suppression of apoptosis and antiapoptotic c-IAP2 gene expression in human monocytic cells. *J Biol Chem.* 2005;280(45):37536-46.
17. Devireddy LR, Green MR. Transcriptional program of apoptosis induction following interleukin 2 deprivation: identification of RC3, a calcium/calmodulin binding protein, as a novel proapoptotic factor. *Mol Cell Biol.* 2003;23(13):4532-41.
18. Soderling TR, Chang B, Brickey D. Cellular signalling through multifunctional Ca<sup>2+</sup>/calmodulin-dependent protein kinase II. *J Biol Chem.* 2001;276(6):3719-22.
19. Fladmark KE, Brustugun OT, Mellgren G, Krakstad C, Bøe R, Vintermyr OK, et al. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II is required for microcystin-induced apoptosis. *J Biol Chem.* 2002;277(4):2804-11.
20. Liu Y, Templeton DM. Cadmium activates CaMK-II and initiates CaMK-II-dependent apoptosis in mesangial cells. *FEBS Lett.* 2007;581(7):1481-6.