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Source / Izvornik: **Arhiv za higijenu rada i toksikologiju, 2009, 60, 449 - 456**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.2478/10004-1254-60-2009-1969

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:163:248928

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Download date / Datum preuzimanja: **2024-04-20**

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DOI: 10.2478/10004-1254-60-2009-1969

Review

A JOURNEY THROUGH MITOGEN-ACTIVATED PROTEIN KINASE AND OCHRATOXIN A INTERACTIONS*

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Received in April 2009
Accepted in November 2009

Ochratoxin A (OTA) is a ubiquitous mycotoxin with potential nephrotoxic, carcinogenic, and cytotoxic action. It has been proposed that OTA might be involved in the development of Balkan endemic nephropathy, which is associated with an increased risk of urinary tract tumours, and of other forms of interstitial nephritis. Cell susceptibility to OTA mainly depends on mycotoxin concentrations, duration of exposure, and intracellular molecular and genetic context. OTA can affect a cell by stimulating or inhibiting certain signalling pathways such as mitogen-activated protein kinase (MAPK). Three major mammalian MAPKs have been described: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. All MAPKs regulate diverse cellular programmes, but in most cases ERKs have been linked to cell survival, while JNKs, and p38 MAPKs have been implicated in cell death by apoptosis. This review looks into OTA-mediated MAPK activation and its effects.

KEY WORDS: *apoptosis, carcinogenicity, kidney, necrosis, oxidative stress, toxicity*

OCHRATOXIN A: A HAZARDOUS MYCOTOXIN

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced by several species of *Aspergillus* and *Penicillium*. OTA has been detected in a variety of animal chow and human food, especially in cereals, wine, coffee, spices, beer, poultry, and pork. The importance of OTA for human health arises from its widespread occurrence and the resulting high risk of exposure. The highest levels of OTA in food were found in Europe, Eastern in particular. However, OTA-containing food was also detected in North and South America, Asia, and Africa (1). The average daily intake of an adult European is 1.044 ng kg^{-1} body weight, although the intake can be as high as 3.55 ng kg^{-1} b.w. (2). Once ingested, OTA is rapidly reabsorbed in the jejunum. In the gastrointestinal tract, OTA is hydrolysed to some extent to a less toxic derivative ochratoxin α (OTα), and 1 % to 4 % of OTA is hydroxylated and conjugated in liver cells. However, these important processes need further clarification (3). According to the European survey conducted by Miraglia et al. (2), mean plasma concentration of OTA in adults is 0.875 nmol L^{-1}, and ranges from 0.53 nmol L^{-1} to 2.75 nmol L^{-1}. OTA half-life in serum ranges from 120 h in rats to 840 h in monkeys and humans (4, 5). This prolonged retention in serum is the consequence of strong binding to serum proteins, mainly to albumin. Therefore, less than 0.2 % of OTA is free and able to leave blood vessels or undergo glomerular filtration. The fraction of OTA bound to proteins constitutes a mobile reserve of OTA that can be released into the tissues as soon

*The subject was presented at the 2nd Croatian Scientific Symposium with International Participation Fungi and Mycotoxins – Health Aspects and Prevention, held in Zagreb, Croatia on 5 December 2008.

as free OTA fraction decreases. Both biliary and renal routes are involved in the excretion of OTA in rats and mice, but the biliary route prevails (6). However, in humans and nonhuman primates (vervet monkeys), OTA is excreted mainly *via* the kidney (7, 8). It is excreted in the kidney tubules by means of organic anion transporters (OATs), and OATs or other transporters mediate its reabsorption in all nephron segments. This delays elimination and increases the risk of OTA accumulation in tissues (9, 10).

OTA has become a human health issue since the suspicion that it is involved in the development of Balkan endemic nephropathy (BEN) (6, 11, 12), a disease associated with an increased risk of urinary tract tumours (13), and the development of other forms of interstitial nephritis (14, 15). Apparently, the aetiology of half the end-stage renal diseases (ESRDs) is unknown, and there is a consensus that mycotoxins such as OTA might play an important role in these cases (16).

In a variety of animal models OTA has produced a wide array of toxicological effects, including nephrotoxicity, nephrocarcinogenicity, neurotoxicity, and immunotoxicity (17, 18).

Numerous studies have tried to elucidate the mechanisms implicated in OTA toxicity (9, 17, 19). The OTA molecule consists of a dihydroisocoumarin moiety that is amide-linked to the *L*-phenylalanine moiety (Figure 1) which is a structural analogue of phenylalanine (Phe). This is why OTA can act on all metabolic systems involving Phe. It was suggested

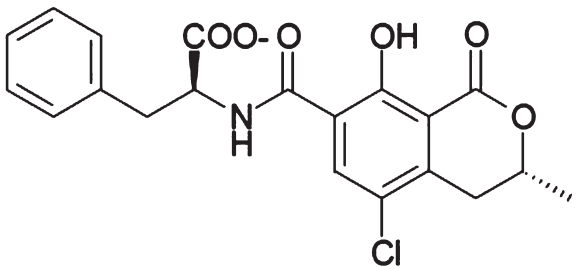

Figure 1 *Molecular structure of ochratoxin A*

that competitive inhibition of Phe-tRNA synthase by OTA is the primary cause of OTA toxicity. It stops amino-acylation and peptide elongation, and disrupts protein synthesis at the post-transcriptional level. Since OTA disrupts protein synthesis, it impairs the activity of several cellular enzymes, and particularly of cytosolic phosphoenolpyruvate carboxykinase, which results in a significant drop in gluconeogenesis. Therefore, an indirect toxic effect of OTA is that it changes carbohydrate metabolic pathways. In addition, the Phe moiety of OTA can bind to and inhibit Phe hydroxylase, and thus impair hydroxylation of Phe to tyrosine, which is the key regulatory step in the catabolism of this amino acid (19). However, all this can not explain the diversity of its toxic effects, such as lipid peroxidation, DNA damage, and disruption of calcium homeostasis. Several studies suggest that OTA toxicity involves oxidative stress (20-23).

It seems that OTA-mediated effects mainly depend on mycotoxin concentrations, duration of exposure (acute and chronic), and the type of the affected cell. As OTA inhibits protein synthesis at relatively high doses, which are not seen in everyday exposure, these effects have only a minor toxicological relevance. However, Gekle et al. (5) have suggested that in the low-dose range (naturally occurring exposure) OTA interacts with key cell targets, changing the signalling pathways. These changes affect the cell function, and may disturb the whole-organism homeostasis. One of these changes is OTA-induced activation of mitogen-activated protein kinases (MAPK) reported by several studies (6, 24-28).

MAPK FAMILY TREE

The MAPK superfamily of serine/threonine kinases is activated by a number of extracellular stimuli, and is involved in signal transduction pathways, that play an important regulatory role in cell survival and death.

A MAPK cascade includes at least three kinases: a MAPK kinase kinase (MKKK), which phosphorylates and activates a dual-specificity MAPK kinase (MKK), which in turn activates MAPK by phosphorylating adjacent threonine and tyrosine residues within their activation loop. Once activated, MAPK can affect a number of cytosolic components through its proline-directed serine/threonine activity, or it can translocate to the nucleus where it can phosphorylate and activate a number of transcription factors involved in immediate early gene expression (29-31). MAPKs are inactivated by a variety of dual-specificity phosphatases called MAP kinase phosphatases or MKPs. Most of the MKPs can act on a number of MAPKs (32, 33). Generally, the antagonistic interplay between a kinase and phosphatase needs to be finely tuned to ensure proper activation of the target protein. It has recently been demonstrated that kinases control signal amplitude, whereas phosphatases

mediate both signal amplitude and signal duration. Reversible phosphorylation of MAPKs emphasizes the importance of the balance between phosphorylating kinases and dephosphorylating phosphatases in regulating these pathways (34, 35).

Three major mammalian MAPKs have been described: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (Figure 2).

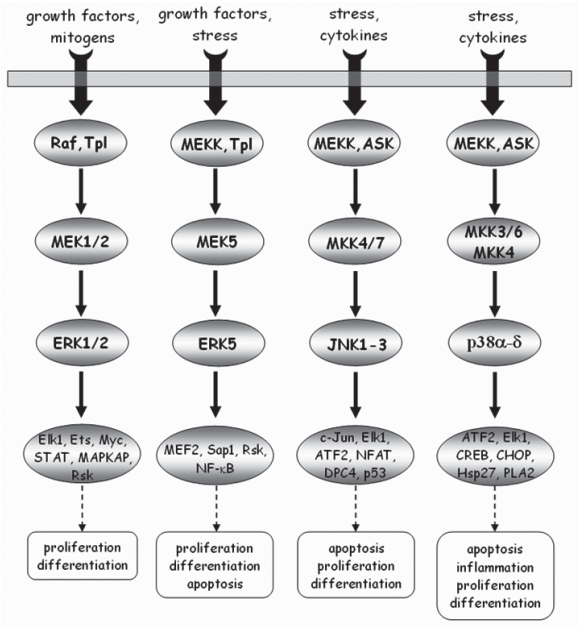

Figure 2 *Biological effects of the MAPK signalling cascade*

The sisters of mercy: ERK1 and ERK2

ERK, the longest known and best characterised member of the MAPK family, has a number of isoforms (ERK1-5 and ERK7/8). By far the most studied are ERK1, 2, and 5 (36). ERK1 and ERK2 are activated mainly in response to growth and differentiation factors. These factors often act through the activation of receptor tyrosine kinases (RTK) that ultimately lead to the activation of the GTP-binding protein Ras, which can bind to and activate serine/threonine kinase Raf (MKKK). Raf in turn phosphorylates and activates dual-specificity kinases MEK1 and MEK2 (MKKs), whose only known substrates are ERK1 and ERK2 (37). ERK2 has been shown to phosphorylate MEK1 *in vitro* (38). Although *in vivo* phosphorylation of MEK1 by ERK2 is yet to be demonstrated, this suggests that ERK2 could participate in its activation in a positive feedback loop. Following dual phosphorylation in the Thr-Glu-Tyr sequence, ERK1 and ERK2 can catalyse the transfer of a phosphate group to a number of cytosolic target molecules such as phospholipase A_2, the tail of the epidermal growth factor receptor, cytoskeletal elements, the MAPK-activated protein kinase-2 and -3 (MAPKAP-K2, MAPKAP-K3), and p90RSK protein kinase, which can translocate to the nucleus and phosphorylate transcription factors. In addition, activated ERK1 and ERK2 can translocate to the nucleus, where they can phosphorylate transcription factors such as Elk-1, SAP-1, c-Myc, and ATF-2 (29-31, 39).

It is crucial that the finely concerted interaction between kinases and phosphatases regulating the ERK1/2 signalling cascade remains undisturbed, as the strength and duration of the signal determine whether the cell will differentiate or proliferate.

The stress managers: JNK and p38

Members of the MAPK superfamily JNK and p38, often jointly referred to as stress kinases, are activated in response to proinflammatory cytokines, growth factors, and various environmental stresses (oxidative stress, UV irradiation, heat, and osmotic shock). Whereas ERK1/2 are primarily involved in cellular survival, JNK and p38 appear to be intimately linked with programmed cell death. JNK and p38 MAPK are activated through two distinct signalling pathways.

JNK family includes three isoforms: JNK1, JNK2, and JNK3. JNK1 and JNK2 are ubiquitous, and JNK3 is more specific to the brain, heart, and testis. The immediate upstream JNK kinases are MKK4 and MKK7. MKK7 preferentially targets threonine 183 and MKK4 phosphorylates tyrosine 185 in the consensus sequence Thr-Pro-Tyr within the JNK activation loop. On the other hand, MKK4/7 are activated by a large group of MKKKs, usually stimulated by GTPases of the Ras or Rho family. After translocation to the nucleus, activated JNK can phosphorylate a number of transcription factors such as c-Jun, ATF-2, and Elk-1, leading to the transcription of immediate early genes that dictate short-term and long-term response to cell stress (40, 41).

The p38 MAPK family has four isoforms, α, β, γ, and δ, differently expressed throughout the organism. p38α and p38β have a wide expression pattern. p38γ is expressed in skeletal muscle, whereas p38δ is detected in small intestine, pancreas, testis, and kidney. MKK4, a well known activator of JNK, has been shown to phosphorylate p38 *in vitro* and when overexpressed *in situ* (42); however, it remains to

be determined if under normal operating conditions p38 is in fact a substrate for MKK4. Activation of MEKK1, the MKK4 upstream regulator, does not lead to the activation of the p38 signalling pathway (38). MKK3 and MKK6 have been identified as essential dual-specificity kinases that phosphorylate threonine and tyrosine residues of the Thr-Gly-Tyr motif of p38. The major cytosolic targets for p38 are serine protein kinases MAPKAP-K2 and MAPKAP-K3, known to activate heat shock protein 27 (hsp27) and cyclic AMP response element binding protein (CREB). In addition, p38 MAPK can translocate to the nucleus where it can phosphorylate several transcription factors such as ATF-2, Elk-1, and Max. p38 has also been linked to the regulation of cytokine production (29, 43).

CLOSE INTERPLAY BETWEEN OTA AND MAPKS

Although it has been reported that possible targets of OTA are the liver, the immune system, and the brain cells, the kidney is the main target of OTA. Chronic OTA exposure leads to impairment of the renal function and morphology and to increased incidence of renal adenoma and carcinoma. Several studies have demonstrated direct association between MAPK-stimulated cell proliferation and renal cancer development (44, 45). A number of organ tumours, including about 50 % of all renal neoplasias, have shown increased ERK1/2 activation (45). On the other hand, JNK might play a role in both tumour growth and tumour suppression (46). Most of the signalling cascades have both pro-survival and pro-death function. This ability to mediate diverse responses depends on factors such as the nature and intensity of the stimulus and cellular context.

It has been shown that OTA may induce cell death. In most cases cells die either by necrosis or apoptosis. Apoptosis is an organised, genetically directed process, also known as *programmed cell death*. Apoptosis allows the organism to control cell number and tissue size, and to protect itself from rogue cells that threaten homeostasis. Changes in gene activity may switch on cell self-destruction programme. Morphologically, programmed cell death is characterised by cell shrinking, membrane blebbing, and eventually fragmentation into membrane-bound apoptotic bodies. During apoptosis, the cell membrane loses its asymmetry, and phosphatidylserine (PS) appears on the cell surface. This is the "eat me" signal for macrophages to clear the body of apoptotic cell without inflammation of the surrounding tissue (47, 48).

In contrast to apoptosis, necrosis has been considered an uncontrolled form of cell death. Morphologically, necrosis is characterised by vacuolization of the cytoplasm, loss of membrane integrity, and cellular swelling. The resulting release of intracellular components into the microenvironment can provoke inflammatory response (47, 48).

Several *in vitro* and *in vivo* studies have suggested that OTA induces necrosis or apoptosis, depending mainly on OTA concentration. In most cases, high OTA concentrations (>1 μmol L^{-1}) cause necrosis of renal cells (both of proximal tubular and collecting duct origin), as demonstrated by lactate dehydrogenase (LDH) release (5, 49). In contrast, at low nanomolar concentrations, OTA stimulates apoptosis, which has been confirmed by chromatin condensation and fragmentation (DAPI staining, haematoxylin/eosin staining), DNA fragmentation (TUNEL assay, diphenylamine method), DNA ladder formation (DNA electrophoresis on agarose gel), and/or apoptotic enzyme caspase-3 activation (5, 26, 28, 50). In addition, at 1 μmol L^{-1}, OTA was associated with both types of cell death (5, 26, 28). These effects also appear to be cell-specific, which might explain why certain epithelial cell types along the nephron differ in susceptibility to OTA.

The exact mechanisms leading to OTA-induced necrosis or apoptosis remain unknown. Horvath et al. (25) have shown that OTA stimulates lipid peroxidation, followed by enhanced membrane permeability. In addition, OTA competitively inhibits some transport proteins and enzymes located in the inner mitochondrial membrane (ATPase, succinate dehydrogenase, and cytochrome c oxidase). These might inhibit mitochondrial respiration and ATP production, leading to ATP depletion within a cell. Both of these actions might contribute to OTA-mediated necrosis. It also seems that cell death by necrosis is associated with impaired macromolecule synthesis caused by OTA concentrations ≥1 μmol L^{-1} (51). OTA-mediated inhibition of protein synthesis may prevent the expression of enzymes involved in defence and repair mechanisms, triggering and/or potentiating other adverse effects of OTA (52).

Changes in the regulation of cell survival contribute to the development of many human diseases. Increased rate of apoptosis in the kidney may lead to polycystic

kidney disease, glomerular sclerosis, or interstitial fibrosis (53-55). These damages were found in porcine nephropathy and BEN. This is why Mantle et al. suggested that apoptosis might play a role in the aetiology of BEN (56).

It has been shown that MAPKs are key components of cell survival/death pathways. Gekle et al. (26, 27) investigated the effects of OTA in two clones of MDCK kidney cells, MDCK-C7 and MDCK-C11, resembling principal cells and intercalated cells of the collecting duct, respectively. In MDCK-C11 cells, nanomolar OTA concentrations provoked primarily necrosis and in MDCK-C7 cells apoptosis. This confirms different sensitivity of cells to OTA. ERK1/2 and JNK1/2 were activated in MDCK-C7 cells, but not in MDCK-C11 cells. The authors suggested that JNK signalling pathway might play a role in OTA-induced MDCK-C7 cell apoptosis.

Some other studies also confirmed OTA-induced MAPK phosphorylation (5, 24, 25, 28). Phosphorylation of ERK1/2 and of their substrates Elk-1 and p90RSK was observed in kidney samples of male Fischer 344 rats fed with a carcinogenic dose of OTA over 7 days, 21 days, and 12 months (24). OTA was found to increase phosphorylation of all three major MAPKs (ERK, JNK and p38) in renal proximal tubule cell lines (OK, NRK-52E and LLC-PK1 cells) (5, 28).

ERK and JNK/p38 activation could exert both protective and adverse effects. However, in most cases ERKs have been linked with cell survival and JNK/p38 with inhibition of cell growth and apoptosis (57). It was suggested that a dynamic balance between the anti-apoptotic ERK pathway and the pro-apoptotic JNK/p38 pathways determines whether a cell will survive or undergo apoptosis (57).

What can also greatly influence a specific biological response is the duration of MAPK activation. Transient ERK activation induced cell proliferation and sustained activation cell differentiation (58). Sustained ERK activation is associated with nuclear translocation and phosphorylation of transcription factors (59, 60). Therefore, in contrast to transient activation, sustained activation might have a different effect on gene expression (58). It has also been reported that the duration of JNK activation is decisive for the cell fate; transient activation leads to cell proliferation or differentiation, and prolonged activation leads to apoptosis (61-63).

The actual molecular mechanism responsible for OTA-mediated activation of MAPK cascades has not yet been elucidated. However, it seems to involve oxidative stress. Reactive oxygen species (ROS) or redox disturbances stimulate redox-sensitive signalling molecules such as MAPKs and modify expression of various genes.

Under normal physiological conditions, apoptosis signal-regulating kinase 1 (ASK1), a kinase that activates JNK and p38 by direct site-specific phosphorylation of their respective upstream kinases, is inhibited by thioredoxin, glutaredoxin, or glutathione-S-transferase (GST)μ, while GSTp inhibits JNK. However, suppression of ASK1 and JNK activities by these molecules is reversed by oxidative stress, which could be induced by OTA. The association between thioredoxin and ASK1 gives rise to a dimer thioredoxin/ASK1, which inactivates ASK1. ROS were found to oxidise thioredoxin reactive thiol groups and to cause disulfide bridge formation within the thioredoxin molecule, which destabilizes the dimer. As a result, ASK1 can escape from thioredoxin inhibition and become multimerised, which corresponds to the active form of the enzyme. Follows downstream activation of JNK and/or p38. ASK1 inhibition by glutaredoxin or GSTμ takes place in the thioredoxin-like manner. In addition, JNK activation is inhibited by its binding to GSTp, and ROS dissociates the GSTp/JNK complex by oxidation of GSTp cysteine residues, leading to formation of GSTp dimers and/or multimers and phosphorylation of JNK (64-66).

On the other hand, it is also possible that OTA affects MAPK activation by downregulating their specific phosphatases through increased ROS production. Protein tyrosine phosphatases, including MKPs, have a conserved cysteine residue within their catalytic domain, and this critical cysteine can be oxidised, leading to a reversible loss of phosphatase function (67).

It must be emphasised that, depending on cell type and doses involved, oxidative stress may lead either to cell death or considerable DNA damage/mutations, which in turn may end in cell transformation and tumour development (68).

In conclusion, a combination of the basic mechanisms of action of OTA (and of active OTA derivatives and conjugates capable to react with cellular macromolecules including DNA) discussed in this review and its long plasma half-life could partly explain the cytotoxicity, genotoxicity, and carcinogenicity of OTA. MAPKs certainly have a role in these processes. However, it would be too pretentious to consider them the most important

players in multiple OTA toxic effects, and most certainly many other molecules, some of them yet to be identified, are contributing to the complex network of OTA action.

Acknowledgements

This work was supported by the Ministry of Science, Education and Sports, Republic of Croatia (grant no. 006-0061245-0977).

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Sažetak

PUTOVANJE KROZ INTERAKCIJE PROTEINSKIH KINAZA AKTIVIRANIH MITOGENIMA I OKRATOKSINA A

Okratoksin A (OTA) posvuda je prisutan mikotoksin za koji se smatra da je potencijalno nefrotoksičan i karcinogen, a može uzrokovati i smrt stanice. OTA se smatra mogućim uzročnikom balkanske endemske nefropatije koju karakterizira povećani rizik od razvoja tumora mokraćnog sustava te različitih drugih vrsta intersticijskog nefritisa. Osjetljivost stanice naspram OTA ovisi ponajprije o koncentraciji mikotoksina, vremenu izloženosti i o unutarstaničnome molekularnom i genskom sklopu. OTA može djelovati na stanicu tako što potiče ili inhibira određene signalne putove u stanici poput puta proteinskih kinaza aktiviranih mitogenima (MAPK). Tri glavne MAPK u sisavaca su proteinska kinaza regulirana izvanstaničnim signalima (ERK), kinaza koja fosforilira N-kraj transkripcijskog faktora c-Jun (JNK) i p38 MAPK. Svi članovi porodice MAPK reguliraju različite stanične programe, s time da ERK najčešće stimuliraju preživljavanje stanica, dok JNK i p38 MAPK najčešće uzrokuju umiranje stanica apoptozom. U ovome smo preglednom članku prikazali na koji način stanice odgovaraju na aktivaciju MAPK koju potiče OTA.

KLJUČNE RIJEČI: *apoptoza, bubreg, karcinogenost, nekroza, oksidacijski stres, toksičnost*

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