The role of protein kinase D during neurotoxic effects

PhD thesis

Written by: Hanna Laura Liliom

Supervisor: Dr. Katalin Schlett, Ph.D., associate professor

Eötvös Loránd University, Faculty of Science

Doctoral School of Biology

Head of the doctoral school: Dr. Anna Erdei, D.Sc., professor

Neurobiology and Anthropology Doctoral Programme

Head of the programme: Dr. László Détári, D.Sc., professor

Neuronal Cell Biology Research Group

Eötvös Loránd University

Department of Physiology and Neurobiology

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Introduction

Stroke is the second most frequent cause of death worldwide. In case of ischemic-type stroke, affected brain regions can be divided into the „core" and *penumbra* areas. The former is more severely affected by loss of perfusion, while within the latter, cell death is delayed. The prevention of this delayed cell death presents an opportunity for therapeutic intervention. The most important underlying molecular mechanisms of delayed neuronal death include excitotoxicity and oxidative stress. Excitotoxicity causes neuronal damage through the overt activation of glutamate receptors. Reactive oxygen and nitrogen species capable of inflicting direct and indirect cell damage cause oxidative and nitrosative stress, respectively, when the equilibrium between their generation and degradation is disrupted (Moskowitz et al., 2010).

Protein kinase D (PKD) enzymes, also termed alternative diacyl-glycerol receptors, constitute a distinct group within the family of serine/threonine kinases. Members of the PKD family can take up various roles within cells and PKD-dependent regulation is highly dependent on cell type and the enzyme's subcellular localisation. PKD activation in response to cytotoxic factors often promotes cell survival. In a well-described PKD activity-dependent protective pathway in non-neuronal cells induced by oxidative stress, survival is mediated by the canonical NFκB signaling pathway (first described by Storz and Toker, 2003). Also in non-neuronal cells, mitochondria were found to be more sensitive to reactive oxygen species in the absence of PKD (Zhang et al., 2015), and PKD activation by oxidative stress was found to induce the phosphorylation of VPS34 kinase, thus exerting a protective effect by regulating autophagy (Eisenberg-Lerner and Kimchi, 2012).

The expression of PKD isoforms in the central nervous system is relatively high and can already be detected early in embryonic development (Oster et al., 2006). In spite of this, information regarding PKD's role in neurotoxic effects is still relatively sparse. An increase in PKD activity in response to oxidative stress has been observed in primary dopaminergic neurons (Asaithambi et al., 2011). In response to *in vitro* ischemia induction, PKD exerts a neuroprotective effect via the phosphorylation of the heat shock protein HSP27 (Stetler et al., 2012). PKD activation was also observed in response to *in vitro* excitotoxicity and *in vivo* focal ischemia. In these circumstances, the neuroprotective role of PKD was also confirmed, which was found to be mediated through the constitutive maintenance of canonical NFκB pathway activity (Pose-Utrilla et al., 2017).

Aims of the study

- Is PKD activity increased in response to oxidative stress induced by acute H_2O_2 treatment or excitotoxicity induced by glutamate treatment in primary cortical neurons?
- In case PKD is activated by oxidative stress or excitotoxicity, does PKD activity exert a positive or a negative effect on neuronal viability?
- In case PKD is activated by oxidative stress or excitotoxicity, which downstream signaling pathway(s) mediate its effects on neuronal viability?
    - Does the cytoprotective oxidative stress / PKD / NFκB signaling pathway described in non-neuronal cells also function in neurons?
    - How does PKD activity or its absence affect mitochondrial functions in neurons?
    - How does the induction of autophagy in oxidative stress- or excitotoxicity-affected cells influence neuronal survival? Is PKD capable of regulating autophagy in neurons, as in non-neuronal cells?

Applied methods

- preparation and maintenance of mouse embryonic cortical neuronal cultures
- induction of excitotoxicity or oxidative stress in cultured neurons through extracellular addition of glutamate or H_2O_2, respectively
- performance of cell viability tests through the MTT assay
- performance of quantitative real time PCR (qRT-PCR) runs
- analysis of the phosphorlyated and total levels of specific protreins via Western Blot, using whole, cytoplasmic or nuclear protein lysates
- transfection of neurons with fluorescently labelled PKD reporter, followed by immunocytochemical detection of the phosphorylated reporter in fixed cortical neuronal cultures
- immunocytochemical detection of the transcription factor RelA (NFκB p65)
- examination of NFκB activity using a luciferase reporter transfected into neurons
- the study of PKD activity-dependent changes in the mitochondrial membrane potential of neurons using TMRM staining

Results

1. The investigation of endogenous PKD activity during neurotoxic effects

The serine group in the 916. position (S916) of PKD is known to be autophosphorylated following enzyme activation, and is therefore used as an indicator of PKD activity (Matthews et al., 1999). The ratio of phosphorylated S916 did not change in response to glutamate treatment-induced excitotoxicity. In contrast, treatment with 50 μM H_2O_2 for 30' already resulted in a significant increase in phosphorylated PKD relative to the total amount of PKD. This effect was maintained for at least 2 hours and died down by the 6. hour of treatment.

To study endogenous PKD activity, we used an EGFP-tagged reporter protein encompassing a phosphorylatable fragment of a known PKD substrate (Czondor et al., 2009). The ratio between the signal intensity of the phosphorylated reporter and EGFP increased after 30' of treatment, but this effect died down significantly by the 1. and 4. hours of treatment.

1.1. Examination of the nuclear translocation of PKD in response to oxidative stress

PKD is known to translocate to the nucleus transiently following its activation (Wang, 2006). To examine this, we analysed the level of total and phosphorylated PKD after H_2O_2 treatment in both nuclear and cytoplasmic protein fractions. The amount of total PKD in the nuclear compartment was increased 1 hour after the start of H_2O_2 treatment. The effect died down by 4 hours of treatment. Phosphorylated PKD levels were unaffected. In cytoplasmic protein fractions, the relative amount of phosphorylated S916 showed a transient increase in response to H_2O_2 (peaking at 2 hours after treatment and dying down by 6 hours of treatment), while total PKD levels remained unchanged.

2. Pharmacological inhibition of PKD increases cell death caused by H_2O_2 treatment

To examine the effect of PKD activity on cell death induced by oxidative stress, neurons were treated with different concentrations (1-4 μM) of a PKD-specific inhibitor (kbNB 142-70) in addition to 24 hours of H_2O_2 treatment. Simultaneous treatment with kbNB 142-70 and H_2O_2 decreased cell viability in a concentration-dependent manner.

3. Analysis of possible downstream pathways mediating PKD effects

3.1. Clarification of the possible role of the NFκB pathway in mediating the effects of oxidative stress-induced PKD activation

Members of the non-canonical NFκB pathway (NIK, p100/p52, RelB), BCL-3 and canonical pathway member c-Rel showed no or only very low mRNA expression in cortical neurons, while the other members of the canonical pathway (IKKα and β, IκBα, a RelA, NFκB p105/p50) were obviously expressed.

We examined the changes in the levels of total and phosphorylated IKKα, IκBα, RelA and NFκB p105/p50 in response to H_2O_2 treatment through Western Blot in cytoplasmic protein samples. The ratio of phosphorylated IKKα showed a slight increase after 2 hours of H_2O_2 treatment. Total protein level remained unchanged until this timepoint and showed a slight decrease at 4 and 6 hours after the onset of treatment. We observed a transient increase in the relative levels of phosphorylated IκBα and RelA already at 30' after the start of the treatment, the effect dying down by 6 hours. Total IκBα levels didn't deviate from control levels throughout the experiment. A steady decrease was seen in the signals of both NFκB p105 and NFκB p50 from the 2-hour timepoint onwards, but we observed no change in the ratio of p105-p50 proteins.

We were unable to show a difference in the distribution of RelA immunostaining between cytoplasmic and nuclear compartments in neurons treated with H_2O_2 for 1 hour, compared to untreated cells. Western Blot experiments showed that the ratio of nuclear to cytoplasmic RelA was decreased in response to 0,5-6 hours of H_2O_2 treatment. The nuclear levels of NFκB p50 also did not change throughout the experiment. In addition to our analyses at the protein level, we utilized a functional reporter assay, during which we monitored the activity of NFκB transcription factor-binding inducibly expressed luciferase enzymes. 100 μM H_2O_2 was found not to change the expression of the enzyme in neurons.

3.1.1. Elucidation of the connection between PKD activity and the canonical NFκB pathway

The PKD-specific inhibitor (kbNB 142-70), added in addition to H_2O_2 to examine the PKD-dependence of the observed NFκB pathway phosphorylation was found not to influence IKKα phosphorylation at the only timepoint of treatment when an increase in relative phosphorylation levels was observed in response to H_2O_2. In the other timepoints, the ratio of phosphorylated protein was either increased or decreased in the presence of kbNB 142-70. In case of IκBα and RelA, the inhibitor also did not change the ratio of phosphorylated proteins.

3.2. Examination of the possible effect of oxidative stress-induced neuroprotective PKD action on mitochondria

We found that active PKD localised to the vicinity of mitochondria. To explore the relationship between endogenous PKD activity and mitochondrial functions in neurons, we examined how the overexpression of an inactive PKD mutant affects the oxidative stress-induced decrease in mitochondrial membrane potential in neurons. 30' H_2O_2 treatment induced a more pronounced decrease in the intensity of TMRM staining in mutant PKD-transfected

neurons, than those transfected with EGFP only (control), in addition to non-transfected neurons.

3.3. Examination of the possible role of autophagy in mediating the neuroprotective effect of oxidative stress-induced PKD activation

3.3.1. The effect of oxidative stress on autophagic activity in cultured cortical neurons

The amount of two typical autophagy marker proteins, SQSTM1/p62 (hereafter referred to as p62) and both forms of LC3B (LC3B-I and LC3B-II) decreased in response to H_2O_2 treatment. When both H_2O_2 and an autophagic degradation inhibitor (bafilomycin) were applied, the signal intensity of p62 and – to a smaller degree – LC3B-II increased compared to both control and H_2O_2-treated samples, indicating their accumulation in the absence of autophagic degradation.

3.3.2. The effects of autophagy-enhancing chemicals (AUTEN67 and AUTEN99) on cultured cortical neurons during oxidative stress

During our collaboration with the research group of Dr. Tibor Vellai (Eötvös Loránd University, Department of Genetics) we examined the effects of chemicals capable of increasing the level of autophagy induction (Papp et al., 2016; Kovacs et al., 2017). Treatment with AUTEN67 only induced a significant decrease in the amount of both p62 and LC3B-II compared to control. AUTEN99 treatment by itself induced the relative reduction of p62 levels only at higher concentrations (25-50 μM). LC3B-II levels, however, remained unaffected. When we also applied inhibitors of autophagic degradation (bafilomycin or chloroquine), LC3B-II and p62 levels increased in comparison to cells treated with the inhibitors only, which indicates their accumulation in the absence of autophagic degradation. This accumulation was more pronounced in case of added AUTEN67 treatment, indicating increased autophagic activity induced by the chemical. According to our results, AUTEN67 enhances autophagy in primary cortical neurons. AUTEN99 is also capable of enhancing autophagy in the examined cell type, albeit with much lower efficiency.

3.3.2.1. The effects of autophagy-enhancing chemicals on the viability of H_2O_2-treated cortical neurons

Addition of 1 or 10 μM AUTEN67 to H_2O_2-treated neurons had a positive effect on their viability. Higher AUTEN67 concentration caused a substantial decrease in viability in these circumstances, however. AUTEN99 had a positive effect on neuronal viability until 5 μM, but concentrations of 10 μM and higher reduced the viability of H_2O_2-treated neurons.

3.3.3. Investigation of the effect of oxidative stress-induced PKD activation on autophagic activity

To examine the connection between oxidative stress-induced PKD activity and autophagy, we applied the PKD-specific inhibitor to cortical neurons treated with H_2O_2 for different durations. We found that the relative level of LC3B-II decreased in H_2O_2-treated neurons in the presence of kbNB 142-70. This decrease was the most pronounced 1 hour after the onset of H_2O_2 treatment. The LC3B-II/LC3B-I ratio showed an even greater decrease in the presence of the inhibitor, indicating that LC3B-II level reduction was coupled with an increase in the amount of LC3B-I.

Conclusions

1. Endogenous PKD activity is increased in response to oxidative stress

Our experimental results indicating PKD activation in response to oxidative stress (an increase in phosphorylated S916, translocation to the nucleus, an increase in reporter phosphorylation) were corroborated by data obtained from live neurons, according to which 30' H_2O_2 treatment increased the activity of a FRET-based PKD activity reporter (Liliom et al., 2017). In conclusion, we have proven via multiple methods that PKD activation is induced by oxidative stress in primary cortical neurons. This conclusion is in concordance with studies performed on non-neuronal cell lines (e.g. Storz and Toker, 2003) and primary dopaminergic midbrain neurons (Asaithambi et al., 2011).

2. Oxidative stress-induced PKD activity has a neuroprotective effect

According to our results, PKD activity has a protective effect on primary cortical neurons, as pharmacological inhibition of PKD during oxidative stress resulted in a greater decrease in viability than that induced by H_2O_2 alone. Other studies also concluded that PKD has protective effects during oxidative stress both in case of non-neuronal (e.g. Storz and Toker, 2003) and neuronal cell lines (e.g. Asaithambi et al., 2011).

3. The identification of candidate pathways mediating neuroprotective PKD activity

3.1. The canonical NFκB pathway is not activated as is widely accepted in response to oxidative stress in neurons

We observed that in response to H_2O_2 treatment: i) IκBα degradation did not occur, ii) neither RelA nor p50 were translocated into the nucleus and iii) the expression of the NFκB reporter luciferase enzyme was unaffected. All of these results indicate that oxidative stress does not induce the activation of NFκB transcription factors according to the canonical pathway

in primary cortical neurons. Keeping in mind the complicated regulation of NFκB transcription factors, it is possible that the NFκB pathway does, in fact, fulfill some regulatory role in primary neurons affected by oxidative stress, as we found increased phosphorylation of canonical signaling pathway members. In our experimental circumstances, however, our results do not support the induction of gene transcription via the canonical NFκB pathway in response to oxidative stress in neurons. This conclusion contradicts several studies performed on neuronal cell types (e.g. Mattson et al., 1997). However, research has been published that also questions the activation of the NFκB pathway in neurons in response to oxidative stress (Listwak et al., 2013).

3.1.1. Active PKD does not directly phosphorylate members of the the canonical NFκB pathway during oxidative stress

In our experiments, we expected a reduction in the ratio of IKKα, IκBα and RelA phosphorylation induced by H_2O_2 treatment in response to the addition of the PKD-specific inhibitor, kbNB 142-70. Instead, the inhibitor did not change the relative phosphorylation level of either protein according to the expected kinetics. Our results indicate that the phosphorylation of the examined NFκB pathway members is dependent on PKD activity, however, this is not performed directly by PKD activated by oxidative stress. Our conclusion contradicts the results of studies working with non-neuronal cells (e.g. Storz and Toker, 2003) and primary neurons (Pose-Utrilla et al., 2017). This contradiction could be explained in theory by e.g. the activity of some other pathway regulated by PKD or a possible aspecific effect of the PKD inhibitor. Further experiments need to be performed, however, to clarify the observed effects.

3.2. The significance of the nuclear translocation of PKD

The increase in the total amount of PKD and the unchanged ratio of phosphorylated PKD in the nuclear protein fraction, taken together, indicate that PKD could partake in transient signaling events within the nucleus, too, in response to H_2O_2. Our results are in agreement with the results obtained from primary dopaminergic midbrain neurons (Asaithambi et al., 2011). Although it has been demonstrated that regulation of neuronal survival can occur through nuclear signaling events dependent on PKD action (Jo et al., 2016), little is known currently about the nuclear activity of neuronal PKD.

3.3. The significance of the mitochondrial association of PKD

We found that endogenous PKD activity and the subcellular localisation of PKD can be observed in the vicinity of mitochondria (Liliom et al., 2017). A similar occurence was found in non-neuronal cells, as well, where PKD was shown to influence the degree of sensitivity of

mitochondria to reactive oxygen species (Zhang et al., 2015). According to our live cell experiment, PKD could be important in the maintenance of mitochondrial functions. Our results do not conclusively show that the neuroprotective effect of PKD is mediated through the enzyme's association with mitochondria. More research is needed in order to clarify this point.

3.4. Autophagy as the possible mediator of the neuroprotective effect of PKD activity

It appears that PKD is capable of influencing autophagic activity in cortical neurons, as in non-neuronal cells, although we have yet to examine whether the enzyme has a role in modulating the activity of proteins directly involved in autophagy regulation. It is a promising result that the decrease of neuronal viability caused by oxidative stress appears to be less severe in case specific autophagy enhancing chemicals are also applied. The few existing studies concerning the putative role of PKD in autophagy have turned out contradictory results, as its effects have been described as both stimulatory (Eisenberg-Lerner and Kimchi, 2012) and inhibitory (Zhao et al., 2017). The connection between PKD and autophagy in neurons has not yet been demonstrated. To clarify whether PKD has a positive or a negative effect on autophagy in neurons requires further experimental work.

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Publications related to the topic of the doctoral thesis

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