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ASSOCIATION OF A-KLOTHO WITH REGULATION OF KEAP1/Nrf2/INTERLEUKIN-1 PATHWAY AND AMPA RECEPTOR TRAFFICKING IN THE BRAIN OF SUICIDE VICTIMS

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Suicide is a significant public health challenge worldwide. Statistical data confirm a strong relationship between suicidal behavior and depressive disorders (DDs), but the molecular mechanisms of these diseases are still poorly understood. A growing body of research suggests that the Klotho-mediated pathway may be a novel intracellular target for the development of suicide-related disorders (including DDs). To verify this hypothesis, the link between α-Klotho levels, Nrf2-related inflammatory status (IL-1α, IL-1β, Keap1, NFκB p65), AMPA (GluA1, GluA2, p-S831-GluA1, p-S845-GluA1) receptor subunit trafficking and AMPK (AMPKα1/2; pT172-AMPKα1) signaling pathways in the brain of suicide victims as compared to controls were investigated. Commercially available enzyme-linked immunoassay (ELISA) and Western blot analysis were performed in the hippocampus (HP) and frontal cortex (FCx) of suicide victims and matched controls. Group differences were assessed using an unpaired Student's t-test. A statistically significant decrease in the level of α-Klotho (HP: p=0.001; FCx: p=0.012) with an increase in IL-1β (HP: p=0.0108) and IL-1α (FCx: p=0.009) concentrations were shown. These alterations were associated with increased Keap1 (FCx: p=0.023) and NF-κB-p65 (HP: p=0.039; FCx: p=0.013 nuclear fraction) protein levels. Furthermore, a significant reduction in p-S831-GluA1 (HP: p=0.029; FCx=0.002) and p-S845-GluA1 (HP: p=0.0012) proteins was observed. Similarly, the level of GluA2 (HP: p=0.011; FCx: p=0.002) and in p-T172-AMPKα1 (HP: p=0.0288; FCx: p=0.0338) protein were statistically decreased. Our findings demonstrate that a reduction in α-Klotho levels in brain structures related to mood disorders (HP, FCx) correlates with suicidal behavior. Moreover, our study provides novel insights into the molecular mechanisms underlying suicide-related disorders, highlighting the role of α-Klotho, Nrf2-related inflammatory status, AMPA receptor trafficking, and AMPK signaling pathways in the pathophysiology of suicidal behavior. These results may have implications for the development of targeted interventions for individuals at risk of suicide.

Key words: *suicide, depression, Klotho, interleukin-1α, interleukin-1β, nuclear factor-κB, Keap1, glutamate receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, adenosine monophosphate-activated protein kinase*

INTRODUCTION

Every year, more than 700,000 people die by suicide. In 2019, suicide accounted for over 1.3% of all deaths, making it the 13th leading cause of death worldwide. Suicide is the fourth (after road injury, tuberculosis, and interpersonal violence) leading cause of death in the 15–29 age group when both sexes are considered together (1, 2).

Due to the fact that suicide represents a global public health problem, attempts are being made to define the most important factors increasing the risk of suicide. Understanding these risk factors is indispensable for the development of an appropriate preventive system. The epidemiology of suicidal behavior is very complex, with certain risk factors including age, sex and ethnic background. However, it is crucial to note that there is evidence linking suicidal behavior and DDs (3, 4, 5). The World Health Organization (WHO) also shows that suicidal behavior is often related to mental disorders such as depression, bipolar disorder (type I, II), schizophrenia, and alcohol use disorders, emphasizing that this behavior and thoughts of suicide are dependent on the type and duration of psychiatric diseases (6, 7).

Suicidal behavior is characterized by a multifactorial pathophysiology. This complex health-related condition involves several alterations at both the central and brain levels

(8). Over the years, mechanisms underlying suicide have been described, such as: the regulation of the hypothalamus-pituitary-adrenal axis (HPA), epigenetic processes, and genetic predisposition (9). Recently, research suggests a crucial role of the association between inflammation and oxidative stress as possible factors predisposing to the development of suicidal behavior (10, 11). Changes in the levels of pro- and anti-inflammatory cytokines or increased oxidative stress parameters have been documented in numerous postmortem studies of suicide victims (12, 13). However, knowledge about intracellular mechanisms involved in this dysregulation is not fully understood and requires further investigation.

Importantly, α-Klotho (Klotho), an anti-aging transmembrane protein, has been linked with regulating tissue inflammatory processes (8-10). Moreover, Klotho increases the anti-oxidant defenses of neural networks (17). It was thus shown that tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-6 (IL-6) downregulate Klotho expression (18,19). Hui *et al.* (2017) found that pretreatment with recombinant Klotho (10 μg/kg, i.v.) inhibited nuclear factor kappa-B (NF-κB) activation and reduced TNF-α and IL-6 levels in mice (20). On the other hand, Klotho's antioxidant properties (increased resistance to oxidative stress) may be linked to promoting the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) (21). Klotho has been shown to activate Nrf2 and provide neuroprotection (due to its anti-inflammatory and antioxidant effects) in neuronal cells. However, the specific molecular mechanism of this action has yet to be determined (17, 22). It should be emphasized that this is currently a new direction of neurobiological research, also assessing the potential role of this regulation in the pathomechanisms of both suicidal behavior and suicide-related disorders (including DDs). Additionally, our previous post-mortem study showed that significantly reduced levels of α-Klotho protein in the prefrontal cortex of patients with major depressive disorder (MDD) were associated with decreased catalase (CAT) and sodium dismutase (SOD) activity. In the same analysis, an increase in IL-6 and TNF-α levels was also observed (23). These results may suggest that Klotho, similar to the Keap1 (Kelch-like ECH-associated protein 1)-Nrf2 system, regulated an NF-κB-dependent mechanism. Both Klotho and Nrf2 suppress NFκB activation, constituting a significant co-regulatory pathway in oxidative and inflammatory responses (24, 25). Gao *et al.* (2022) suggest that the NFκB p65 subunit can directly inactivate Nrf2 *via* competition of the CH1-KIX domain of CREB binding protein (CBP) (26). Nrf2 upregulation in memory mechanisms was also linked with suppression of NFκB and activation of CREB and IRS/PI3K/Akt/ GS3Kβ axis (27). Likewise, Klotho induces neuroprotective effects through PKA/CaMKII/CREB signaling (28). A possible association between Klotho and AMP-activated protein kinase (AMPK; a master sensor and regulator of energy balance) has been demonstrated (29). Furthermore, increasing evidence indicates that AMPK activation promotes anti-inflammatory responses in microglia exposed to various stressors, primarily through inhibition of NFκB signaling and activation of the Nrf2 pathway (30).

Another promising molecular approach in the reduction of inflammation and oxidative stress is the evaluation of the role of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subunit trafficking in the Klotho-Nrf2 pathway (31). AMPAR-mediated neuronal activity of astrocytic Nrf2 signaling and regulation of Klotho expression in synaptic plasticity has been demonstrated (32-34). AMPAR subunit alterations were also observed in the brains of suicide victims (11, 35-39). So, suppose AMPAR may be involved in suicidality. In that case, assessing the mechanisms regulating this pathway is justified, especially because numerous studies have shown contradictory results, such as differences in the level of changes and dependence on brain regions. Our review also highlights the importance of regulating the Klotho-Nrf2 pathway in conjunction with the glutamatergic neurotransmission (14). Furthermore, the role of Klotho was shown in the antidepressant and anti-suicidal effects of low-dose ketamine [a non-competitive channel blocker of N-methyl-D-aspartate receptor (NMDAR)] infusion in patients with treatment-resistant depression (TRD) and suicidal ideation (15).

Given the arguments that the Klotho-Nrf2-AMPAR pathway may be involved in inflammation regulation, this study assessed, for the first time, the changes in α-Klotho levels, Nrf2-related inflammatory status (IL-1α, IL-1β, Keap1, NFκB p65), AMPA (GluA1, GluA2, p-S831-GluA1, p-S845-GluA1) receptor subunit trafficking and AMPK (AMPKα1/2; pT172-AMPKα1) signaling in the hippocampus (HP) and frontal cortex (FCx), a two key brain structure in the development of stress-related disorders including suicidal (3) of suicide victims.

MATERIALS AND METHODS

Informed consent statement

All the tissues used in the present study were collected (in the period 2001–2004) during an autopsy (in Poland referred to as the forensic medical autopsy), which takes place at the request of the prosecutor (without the consent of the patient or closest relatives), to determine the cause of death (legal basis: Article 209 Code of Criminal Procedure of 6 June 1997). During an autopsy, it is permissible to collect tissues for research, however, at that time (in Poland), there was no legal regulation regarding the use of such tissues for research. Therefore, people interested in collecting these tissues and using them in scientific research applied (before storing and using tissues) to the Ethics Committee (legal basis: regulation of the minister of health and social welfare of May 11, 1999 on detailed rules for the appointment and financing as well as the mode of operation of bioethics committees) at the Institute of Pharmacology of the Polish Academy of Sciences in Cracow for approval for such activities. The Ethics Committee agreed (on July 20, 2000) to collect tissues and use them in research. The use of these tissues for research is also compliant with current legal regulations (Article 4 and 5, ACT of July 1, 2005 on the collection, storage and transplantation of cells, tissues and organs). In summary, the use of human material in this study (despite the lack of express consent of individual patients or their closest relatives) was consistent with Polish law and the code of ethics (both in 2001–2004 period and currently in force).

Human subjects and tissue processing (Fig. 1)

Post-mortem brain tissues [HP - CA1 region and FCx - Brodman Area 10] from 14 suicide victims and 8 sudden death controls (mean age ± SEM; 31±4.89 and 29.2±3.59 years, respectively) were collected during the autopsy carried out at the Department of Forensic Medicine, Jagiellonian University Medical College (grant no. 6P05B 142 20 from the State Committee for Scientific Research, approved by Ethics Committee (2001-2004). All the dissected tissues were portioned, frozen, and stored at −80ºC until the start of scheduled analyses.

The ethics committee approved a waiver of informed consent for this study, bearing in mind that none of the study participants would be identified (we only have data disclosed in our previous reports) (12, 40), and consented to both the collection of tissues and their use in this study (approval

number 7/2000 of July 20, 2000). Based on the available medical history, it was found that both the control group and the suicides included in this study were not treated for any chronic diseases of the central nervous system. Among the suicides, 3 deaths were caused by drug overdoses, but it is known that these people were not taking any medications, including psychotropic ones, on a regular basis. The causes of death, age and gender of the subjects included in the study are presented in *Table 1*.

α-Klotho and cytokines assessments

α-Klotho and interleukins concentrations were measured in the whole tissue lysates using commercially available enzyme-linked immunoassay (ELISA) assays (BT LAB, Shanghai, China for Klotho and RayBiotech, Norcross, GA, USA for cytokines). Briefly, samples were prepared, pipetted, and incubated with appropriate reagents according to the manufacturer's protocols. All standards and samples were run in duplicate. The

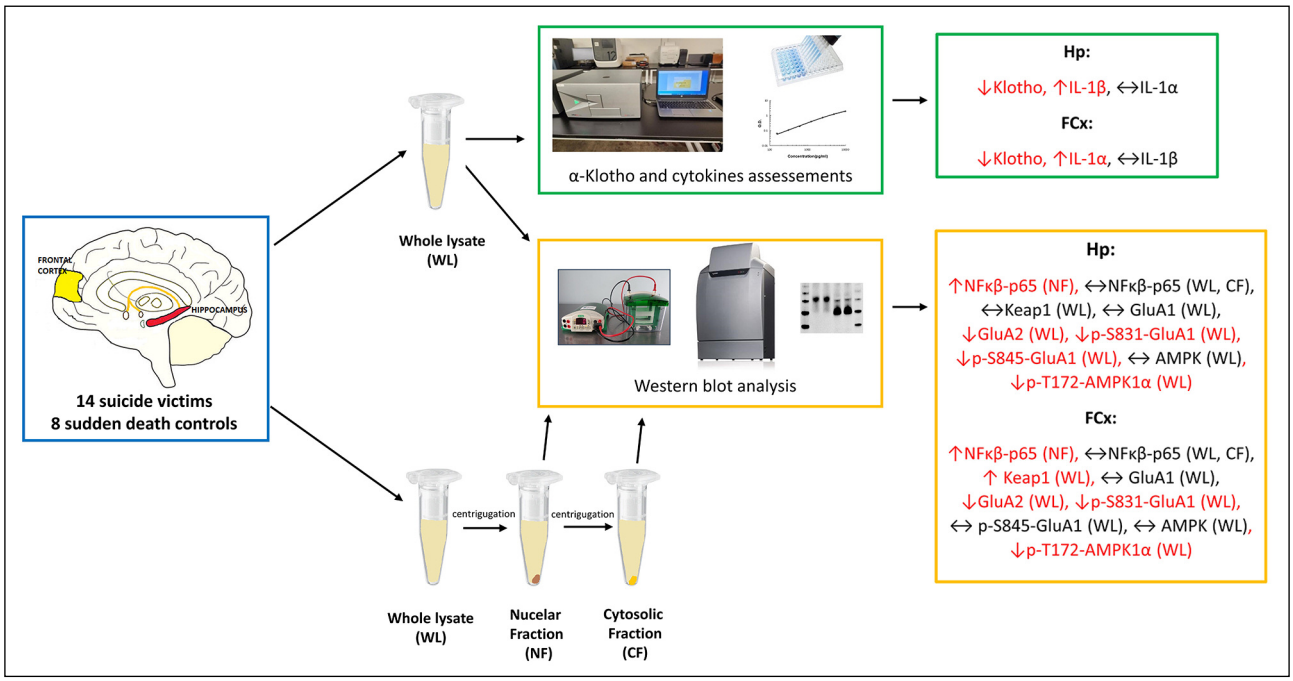

Fig. 1. Flow chart of analyses and major outcomes of this study.

Table 1. Demographic characteristics of controls and suicide subjects. Based on (12, 40).

	Age (years)	**Sex**	**Cause of death**	**Age** (years)	**Sex**	**Cause of death**
	CONTROLS			**SUICIDES**		
	17	M	Cranial/brain injure	33	M	Hanging
	44	F	Road accident	29	M	Hanging
	20	M	Carbon monoxide poisoning	21	F	Hanging
				17	M	Hanging
	54	M	Myocardial infarction	47	M	Hanging
	29	M	Homicide	19	M	Jump under train
	42	M	Myocardial infarction	21	F	Self-poisoning/drug overdose (doxepine + clomipramine)
	21	F	Homicide			
	21	F	Road accident	29	F	Jumping
				20	M	Self-poisoning/drug overdose (hydroxyzine + perazine)
				55	M	Hanging
				20	F	Self-drowning
				55	F	Self-poisoning/drug overdose (diazepam + ethanol)
				24	M	Jumping
				19	M	Hanging
Mediana	*25.0*			*22.5*		
Mean	*31.0*			*29.2*		
SEM	*4.89*			*3.59*		

M, male; F, female

concentration of the tested proteins was detected colorimetrically using a TECAN Infinite 200 microplate reader at a wavelength of 450 nm and calculated according to the manufacturer's recommendations. The range of the measured concentrations was 2.47–1000 pg/ml, 0.5–300 pg/ml, and 0.3–100 pg/ml for α-Klotho, IL-1α, and IL-1β, respectively. All obtained values were normalized to total protein concentration.

Western blot analysis

Western blot analysis of the following proteins: Keap1, NFκB p65, GluA1, GluA2, p-S831-GluA1, p-S845-GluA1, AMPKα1/2, and p-T172-AMPKα1 was performed in the whole tissue lysates. Furthermore, NFκB p65 protein was also analyzed in the cytosolic and nuclear fractions.

First, all the human brain samples were prepared according to the protocols described previously (12). Subsequently, the concentration of total protein in both whole tissue lysates and cell fractions was determined using a PierceTM BCA Protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). Finally, samples containing 50 μg or 20 μg (whole tissue lysates and tissue fractions, respectively) of total protein and loading buffer were electrophoretically separated on an 8–10% (depending on the molecular weight of the tested protein) SDS-polyacrylamide gel and transferred to nitrocellulose membranes (pore size 0.45 μm; Bio-Rad, Feldkirchen, Germany). After blocking (1% blocking solution; BM Chemiluminescence Western Blotting Kit (mouse/rabbit); Roche, Basel, Switzerland) and overnight incubation at 2–8ºC with specific primary antibodies: rabbit polyclonal anti-NF-κB-p65, 1:100; rabbit polyclonal and anti-Keap1, 1:1000; rabbit polyclonal anti-GluA1, 1:1000 Abcam, Cambridge, UK; rabbit monoclonal anti-GluA1 phosphorylated on Serine-831 (p-S831-GluA1), 1:1000, Abcam, Cambridge, UK; rabbit monoclonal anti-GluA1 phosphorylated on Serine-845 (p-S845-GluA1), 1:1000, Abcam, Cambridge, UK; rabbit polyclonal anti-GluA2, 1:1000, Abcam, Cambridge, UK; rabbit polyclonal anti-AMPKα1/2, 1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; rabbit polyclonal anti-AMPKα1 phosphorylated on threonine-172 (p-T172-AMPKα1), 1:200, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; (*Table 2*), the membranes were washed in Tris-buffered saline with 0.1% Tween20 (TBS-T). Proteins were developed by using an

Table 2. The list of primary antibodies (with regard to the applied dilutions) used in the study.

Antibody	**Host species**	**Clonality**	**Cat. number**	**Dilution factor**	**Company**
anti-GluA1	Rabbit	Polyclonal	ab31232	1:1000	Abcam, Canbridge, UK
anti-pS831-GluA1	Rabbit	Monoclonal	ab109464	1:1000	Abcam, Cambridge, UK
anti-pS845-GluA1	Rabbit	Monoclonal	ab76321	1:1,000	Abcam, Cambridge, UK
anti-GluA2	Rabbit	Polyclonal	ab20673	1:000	Abcam, Cambridge, UK
anti-AMPKα1/2	Rabbit	Polyclonal	sc-25792	1:200	Santa Cruz Biotechnol., Dallas, TX, USA
p-T172-AMPKα1	Rabbit	Polyclonal	sc-33524	1:200	Santa Cruz Biotechnol., Dallas, TX, USA
anti-NFkB-p65	Rabbit	Polyclonal	ab16502	1:1000	Abcam, Cambridge, UK
anti-Keap1	Rabbit	Polyclonal	32450	1:1000	SAB-Signalway Antibody, Greenbelt, MD, USA
anti-β-actin	Mouse	Monoclonal	A5441	1:10000	Sigma-Aldrich, St. Louis, MO, USA

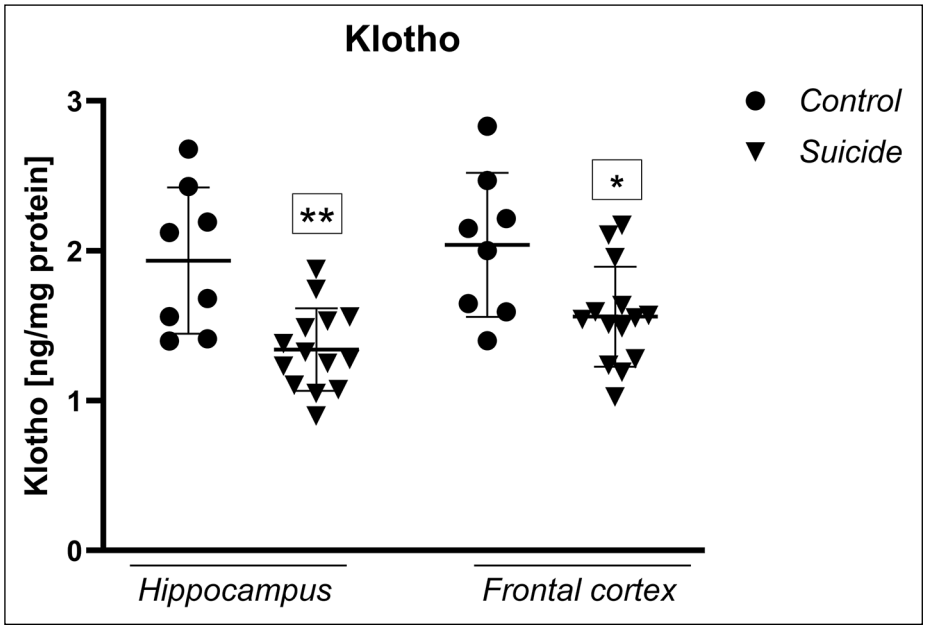

Fig. 2. Reduced α-Klotho concentration (assessed by ELISA) in both the hippocampus ($p<0.01$) and frontal cortex ($p<0.05$) of suicides (n=14) in relation to the sudden death controls (n=8). The values represent the mean ±SD and were analyzed by unpaired Student's t-test. *$p<0.05$; **$p<0.01$ relative to Control.

appropriate secondary antibodies (goat anti-rabbit/anti-mouse IgG peroxidase-conjugated antibodies; dilution: 1:20,000; Bio-Rad, Feldkirchen, Germany) and enhanced chemiluminescence reaction (Clarity Western ECL Substrate; Bio-Rad, Feldkirchen, Germany). Signals were visualized using Syngene G-Box Chemi XT4 and quantified using GeneSys software. The optical density of each protein band was normalized to the density of the β-actin (mouse monoclonal ani-β-actin, 1:1000, Sigma, Germany) (*Table 2*) band (transfer and loading control).

Statistical analysis

Data was evaluated using GraphPad PRISM software ver. 10.0, San Diego, CA, USA). ELISA results are expressed as ng of tested proteins/mg of total protein. Western blot results are presented as GluA1, p-S831-GluA1, p-S845-GluA1, GluA2, AMPKα1/2, p-T172-AMPKα1, NF-κB-p65 and Keap1/β-actin ratio. All results are presented as means ±SD (standard deviation). The Shapiro-Wilk test was performed in order to evaluate the normal distribution of quantitative data. To assess the equality of variances of the studied groups, Levene's test was used. Because all data showed the normal distribution and homogeneity of variances, group differences were assessed using an unpaired Student's t-test, $p<0.05$ was considered as statistically significant.

RESULTS

Elevated level of interleukins accompanied by a decrease in the level of α-Klotho in suicide victims

As shown in *Fig. 2*, α-Klotho levels were significantly lower in both HP [↓30%, $t(20)=3.674$, $p=0.001$] and FCx [↓25%, $t(20)=2.757$, $p=0.012$] of the suicide victims) compared to sudden death subjects. Perason's correlation did not reveal any correlations between Klotho concentration in both examined brain structures and the age of the study subjects [Hp: $r<0.09$; FCx: $r<0.28$; correlation plots not presented]. At the same time, a statistically significant increase in the level of IL-1β [↑82%, $t(20)=2.811$, $p=0.0108$] (*Fig. 3B*) and a clear upward trend in IL-1α [$t(20)=1.995$, $p=0.060$] (*Fig. 2A*) were observed in HP of suicides. Moreover, an increase in the concentration of IL-1α

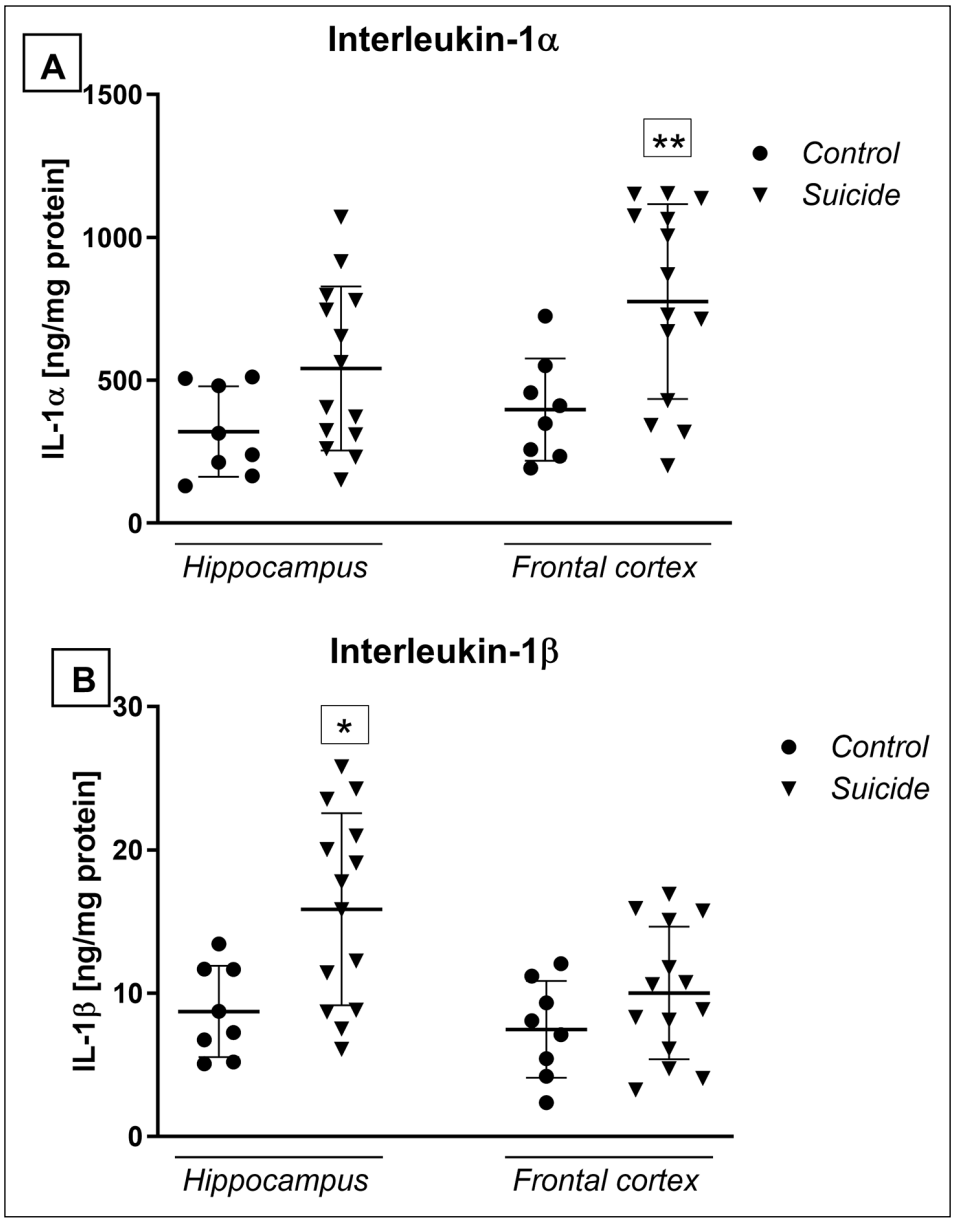

Fig. 3. Alterations in the interleukin-1α (A) and interleukin-1β (B) concentrations (assessed by ELISA) in the hippocampus and frontal cortex of suicides (n=14) in relation to the sudden death controls (n=8). Significantly higher levels of IL-1α were noted in FCx ($p<0.01$), while elevated levels of Il-1β were found in HP ($p<0.05$) of suicides. The values represent the mean ±SD and were analyzed by unpaired Student's t-test. *$p<0.05$; **$p<0.01$ relative to Control.

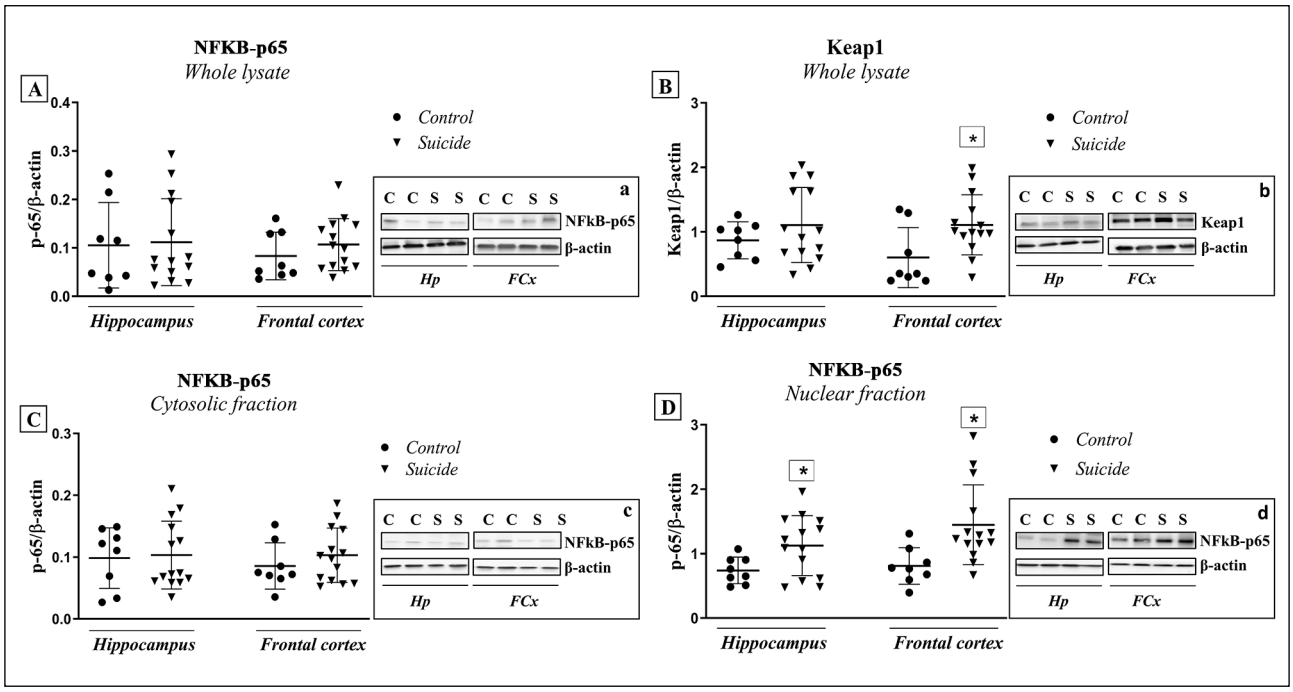

Fig. 4. Alterations in the NFκB-p65 and Keap1 protein levels in the whole lysates (A and B, respectively) and NFκB-p65 protein level in the cytosolic (C) and nuclear (D) fractions (assessed by Western blot) of the hippocampus and frontal cortex of suicides (n=14) compared to the sudden death controls (n=8). Keap1 levels in whole lysates of FCx (but not HP) were higher in suicides ($p<0.05$). In the nuclear fraction of both HP and FCx of suicides, NFκB-p65 levels were significantly ($p<0.05$) increased, while in whole lysates and cytosolic fractions did not differ significantly compared to the control group. The values (mean ±SD) represent normalized (to β-actin) optical density and were analyzed by unpaired Student's t-test. *$p<0.05$ relative to Control. Note: due to significant differences in band intensities (HP vs. FCx) in *Fig. 4B* and *4D*, the values in HP have been multiplied *10 to enable comparison of the data distribution. Right panels present immunoblots of NFκB-p65 (a, c, d) and Keap1 (b) with corresponding β-actin from representative subjects used in the analysis. *Abbreviations:* C, control; S, suicide.

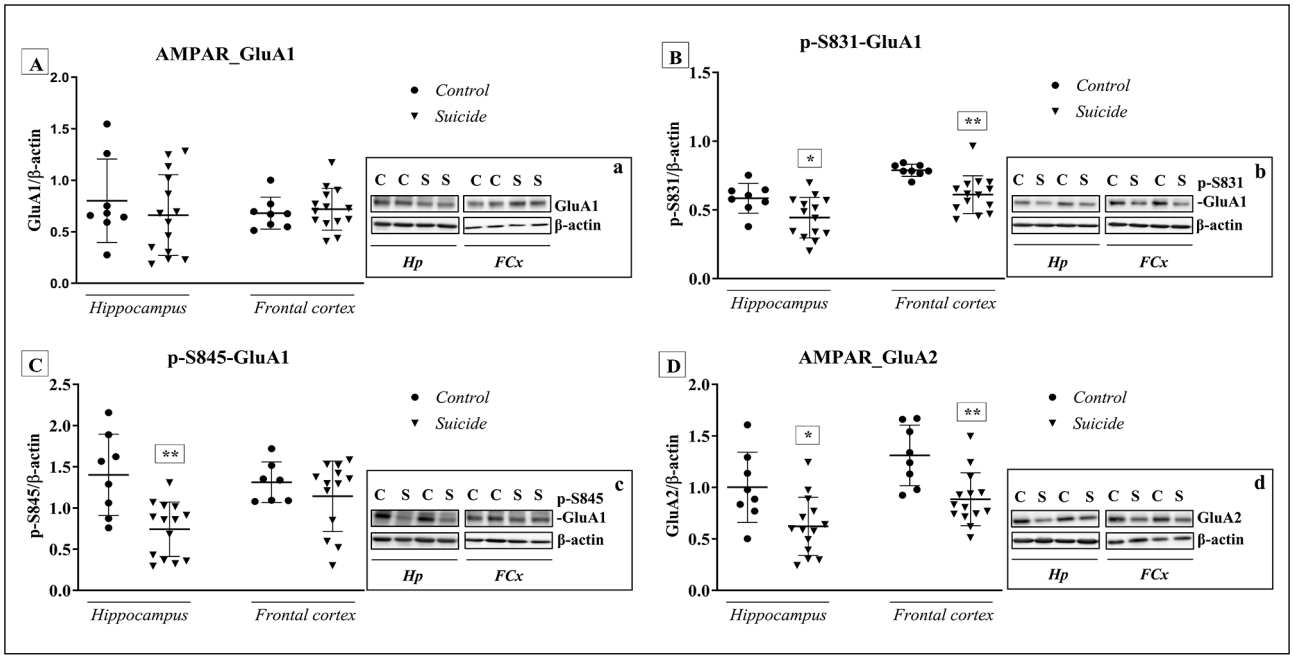

Fig. 5. Alterations in the GluA1 (A), p-S831-GluA1 (B), p-S845-GluA1 (C) and GluA2 (D) protein levels (assessed by Western blot) in the whole lysates of the hippocampus and frontal cortex of suicides (n=14) compared to the controls (n=8). Significant decrease in p-S831-GluA1 levels in HP ($p<0.05$) and FCx ($p<0.01$) as well as decrease in p-S845-GluA1 in HP ($p<0.01$) of suicides were noted. The total GluA1 levels remained unchanged, while the levels of total GluA2 in both brain structures examined were decreased (HP: $P<0.05$; FCx: $p<0.01$). The values (mean ±SD) represent normalized (to β-actin) optical density and were analyzed by unpaired Student's t-test. *$p<0.05$; **$p<0.01$ relative to Control. Right panels present immunoblots of GluA1 (a), p-S831-GluA1 (b), p-S845-GluA1 (c) and GluA2 (d) with corresponding β-actin from representative subjects used in the analysis. *Abbreviations:* C, control; S, suicide.

[↑95%, t(20)=2.899, p=0.009] (*Fig. 3A*) and no change in the level of IL-1β [t(20)=1.360, p=0.1891] (*Fig. 2B*) was noted in FCx of suicides compared to the control group.

Altered protein levels of the intracellular Keap1-Nrf2 pathway

Quantitative results and representative immunoreactive bands corresponding to Keap1 (60 kDa) and NF-κB-p65 (65 kDa) and adequate β-actin (42 kDa) in the whole tissue lysates as well as cytosolic and nuclear fractions (NF-κB-p65) are presented in *Fig. 4*.

Western blot analysis showed a significant increase in Keap1 protein levels (*Fig. 4B*), but only in FCx [↑109%, t(20)=2.461; p=0.023] of suicide victims as compared to the control group. Furthermore, no significant changes in NF-κB-p65 protein levels were noted in the whole lysates [HP: 0.115±0.024 vs. 0.105±0.031, t(20)=0.158; p=0.876] [FCx: 0.107±0.014 vs. 0.083±0.019, t(20)=1.021; p=0.319] (*Fig. 4A*) and cytosolic fraction [HP: 0.103±0.014 vs. 0.098±0.017, t(20)=0.207; p=0.838] [FCx: 0.103±0.013 vs. 0.087±0.012, t(20)=0.940; p=0.358] (*Fig. 4C*) obtained from brain of suicides. On the contrary, in the nuclear fraction of FCx and HP of suicide victims, a statistically significant increase in the level of NF-κB-p65 protein [FCx: ↑79%, t(20)=2.740; p=0.013; HP: ↑52%, t(20)=2.209; p=0.039] (*Fig. 4D*) was revealed.

Decreased level of the GluA2 subunit and altered phosphorylation profile of the GluA1 subunit and AMPK in the suicide victims

Quantitative results and representative immunoreactive bands corresponding to the studied proteins and adequate β-actin (42 kDa) in the whole tissue lysates are presented in *Fig. 5* [GluA1 (102 kDa), p-S831-GluA1 (102 Da), p-S845-GluA1 (102 kDa) and GluA2 (99 kDa)] and *Fig. 6* [AMPKα1/2 (63 kDa) and p-T172-AMPKα1 (63 kDa)].

The level of GluA1 subunit in suicide victims both in HP (0.662±0.105) and FCx (0.240±0.018) were not statistically different from the control group [(0.801±0.143; t(20)=0.792, p=0.438) and (0.227±0.018; t(20)=0.455, p=0.653), respectively] (*Fig. 5A*). On the other hand, significant reduction in p-S831-GluA1 [by ~27%, t(20)=2.347, p=0.029 in HP and ~22%, t(20)=3.511, p=0.002 in FCx; *Fig. 5B*] and p-S845-GluA1 proteins [by ~49%, t(20)=3.768, p=0.0012 in HP; *Fig. 5C*] in the suicides compared to the matched controls were observed. Similarly, the level of GluA2 protein in suicides was statistically different from the sudden death control group [0.622±0.075 vs. 1.001±0.121; t(20)=2.809, p=0.011 in HP and 0.884±0.069 vs. 1.310±0.104; t(20)=3.550, p=0.002 in FCx] (*Fig. 5D*).

On the contrary, the levels of total AMPKα1/2 in suicide victims both in HP (0.851±0.070) and FCx (0.526±0.029) were not statistically different from the control group [(0.804±0.111; t(20)=0.377, p=0.701) and (0.512±0.040; t(20)=0.277, p=0.784), respectively; *Fig. 6A*]. Simultaneously, significant reduction in p-T172-AMPKα1 protein (by 31.41%, t(20)=2.347, p=0.0288 in HP and 19.19%, t(20)=2.276, p=0.0338 in FCx) in the suicide group with regard to controls was observed (*Fig. 6B*).

DISCUSSION

Suicide and suicidal behavior represent complex and multifactorial medical conditions (5). The inflammatory impact appears to be important in the pathogenesis of suicide (10).

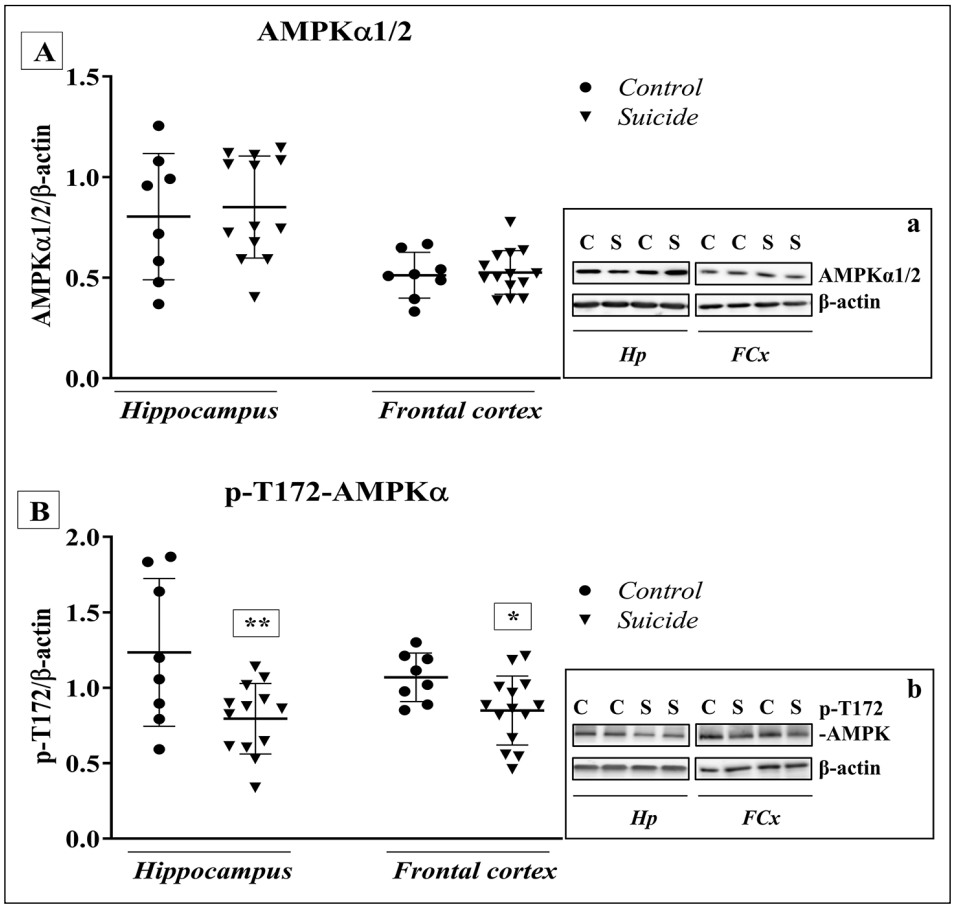

Fig. 6. Reduced p-T172-AMPKα protein levels (assessed by Western blot) in the whole lysates of the hippocampus ($p<0.01$) and frontal cortex ($p<0.05$) of suicides (n=14), with unchanged total AMPKα1/2 protein levels compared to the controls (n=8). The values (mean ±SD) represent normalized (to β-actin) optical density and were analyzed by unpaired Student's t-test. *$p<0.05$; **$p<0.01$ relative to Control. Right panels present immunoblots of AMPKα1/2 (a) and p-T172-AMPKα (b) with corresponding β-actin from representative subjects used in the analysis. *Abbreviations:* C, control; S, suicide.

Immune system abnormalities associated with specific pro-inflammatory cytokine profiles have been observed in suicide victims (41). Interestingly, a growing body of evidence strongly supports the hypothesis that the anti-aging protein α-Klotho is involved in modulating tissue inflammatory responses (42, 43).

Our post-mortem analysis, for the first time, showed significantly decreased α-Klotho protein levels in brain tissues (HP and FCx) of suicide victims. Recent studies have linked Klotho downregulation to the development and progression of DDs (44). For example, Gao *et al.* found reduced levels of plasma α-Klotho in the patients with MDD compared to controls (16). Moreover, our previous study indicated decreased α-Klotho levels in the prefrontal cortex (Brodmann area 10, BA-10) of MDD (23). Some results also suggest that Klotho may be regulated by different treatment options for depression (15, 45). Hoyer *et al.* show that electroconvulsive therapy enhances Klotho in the cerebrospinal fluid of MDD patients (46). In turn, Sartorius *et al.* observed no effects of electroconvulsive or antidepressant (escitalopram, venlafaxine, bupropion) therapy on soluble α-Klotho serum levels in patients with MDD (47). In the context of Klotho, only one published paper found no association between changes in serum Klotho levels and suicidal symptoms in treatment-resistant depression (TRD). Simultaneously, the authors did not exclude the role of Klotho in the antisuicidal effects of ketamine (low-dose; 0.5 mg/kg) (9). Following reports of DDs, our findings indicate that α-Klotho deficiency in the central nervous system may underlie suicidality. Furthermore, in the suicidal group, α-Klotho level changes were associated with increased IL-1α and IL-1β concentrations in the FCx and HP, respectively. Interleukin-1 family members are major intracellular regulators of innate immune processes (48). Limited post-mortem studies have proven an increase in IL-1 levels in individuals with suicidal behavior. Pandey *et al.* show significantly elevated IL-1β expression (mRNA, protein) in the prefrontal cortex (BA10) of teenage suicide victims compared with controls (49). In contrast, the presented study showed enhanced IL-1β concentrations in the HP, but not in the FCx of suicides. However, it is important to emphasize that the group of suicide victims in Pandey *et al.* post-mortem analysis were young people (n=24; mean age 16.26±2.03), which may have a decisive influence on different profiles of changes (49). Similar to our results, Tonelli *et al.* also showed no significant difference in IL-1β (mRNA expression) between controls and suicides in the orbitofrontal cortex area (50). On the other hand, some studies reported a decrease in cytokine levels in the suicidal group. For example, Lu *et al.* showed a decrease in plasma IL-1β concentrations in suicide and control subjects (51). These discrepancies may be due to the outcome of type and sample size, study protocols, and factors such as medication history, post-mortem tissue parameters, or types of suicidal behavior. An additional intracellular mechanism for IL-1 regulation may also be an important determinant of the final changes. Currently, in the regulation of neuroinflammation, the role of Klotho in the NLRP3 inflammasome with Keap1-Nrf2 signaling pathway activation has been suggested (52). It has been demonstrated that Klotho suppressed activation of the NLRP3 inflammasome in macrophages mainly through enhancing fibroblast growth factor 23 signaling (FGF23) (53). The NLRP3 complex is critical for the regulation of IL-1β production (conversion of pro-IL-1β to active IL-1β) (54). Numerous studies have indicated that non-psychological NLRP3 activation enhances IL-1β secretion and contributes to the development of depressive symptoms (55-57). Pandey *et al.* found an increased level of NLRP3 (mRNA, protein) prefrontal cortex (BA 9) collected from people who died by suicide (58). Another possible mechanism that explains the function of Klotho in neuroinflammation is associated with the suppression of NFκB activation (25). Increased NFκB expression (mRNA) has been correlated with the downregulation of Klotho in hippocampal tissue samples from patients with temporal lobe epilepsy (59). Our results also indicate that the downregulation of Klotho in both FCx and HP was linked to significantly increased nuclear levels of NFκB p65 (known as RelA, a major transcriptionally active subunit of cytokine-induced NF-κB).

Importantly, this signaling pathway may be mediated by Nrf2-related antioxidant responses (60). A previous post-mortem study showed a significant reduction in Nrf2 protein levels in the nuclear fraction of FCx in the suicidal group (12). Currently, results show an increase in a major negative regulator of Nrf2-Keap1 protein level in this brain area. Taken together, our results suggest that Klotho may be involved in the regulation of NFκB and Nrf2 signaling pathways. The proposed hypothesis of the impact of the Klotho-NFKB-Nrf2 cascade in the pathogenesis of suicidality requires further investigation. The possible consistent Klotho-Nrf2-IL1 profile of changes observed in depressive disorders is important, as it also appears to be involved in the development of suicidal behavior. However, it is necessary to determine the regulation mechanisms only for suicide victims (non-psychiatric diagnosed) and compare them with the suicidal behaviors that occur in DDs.

An important observation in our study is the reduction of the GluA2 and the phosphorylated forms (p-S831 and p-S845) of the GluA1 subunits of AMPAR in both HP and FCx, which allows us to assume that a glutamate-dependent mechanism may be important in the regulation of molecular pathways related to Klotho and Nrf-2 in suicidal behavior. Previous studies in the brains of suicide victims indicate some changes in AMPAR, but these data are not comprehensive or consistent. For example, increased AMPAR binding has been reported in the caudate nucleus or amygdala of suicide subjects (61-63). On the contrary, other studies have shown attenuation of AMPAR-related transduction pathways (*e.g.*, p44/42 MAPK activity) in depressed suicide patients (34). The importance of some polymorphisms in genes encoding AMPAR subunits in suicidal behavior was also discussed (64, 65). Many more reports concern the importance of AMPAR in MDD patients, who showed various changes in the subunits' expression (including GluA1 and GluA2). Although these studies are much larger, not all of them are consistent with each other and fully consistent with our observations (66-69). Numerous animal studies also confirm the important role of AMPAR in both the pathomechanism of DDs and their treatment (69). Particular importance is attributed to numerous post-translational modifications *e.g.*, phosphorylation at the AMPAR GluR1 subunit serine 831 (S831) and S845 sites, resulting in changes in AMPAR-mediated synaptic currents. Phosphorylation of AMPAR subunits is an important factor determining the transport of receptors to the cell membrane (receptor trafficking), where they function, and then to sites of their degradation, as well as within the cell membrane (between the synaptic and extrasynaptic spaces) (70). All this critically affects the number of active receptors in the cell membrane. The first to be noticed was S831, *i.e.*, a site phosphorylated by protein kinase C (PKC) and CaMKII, which increases its phosphorylation after long-term potentiation (LTP). Then, it demonstrated that the activity-dependent synaptic trafficking of GluR1 depends on S845, *i.e.*, the site phosphorylated by protein kinase A (PKA). The requirement for S845 seems to be for targeting GluR1 to the cell membrane and thus is suggested to prime AMPAR for synaptic trafficking. Dynamic activity-dependent alterations and AMPAR trafficking are thought to underlie changes in synaptic strength and affect cognitive processes (71-73). Klotho deficiency was shown to affect long-term plasticity (usually *via* postsynaptic mechanisms) (74). This may suggest a direct effect of Klotho on

the glutamatergic receptor pool. Dubal *et al.*, showed that increasing Klotho levels prevented the depletion of NMDA receptor (NMDAR) subunits in HP and enhanced spatial learning and memory in mice. Upregulation of Klotho increased the abundance of the GluN2B NMDAR subunit at postsynaptic densities and long-term NMDAR-dependent potentiation (34), but the NMDAR/AMPAR ratio was not significantly different (72). Importantly, this study did not verify changes in individual AMPA receptor subunits, which may be of key importance for the final conclusions. In this aspect, our results seem very important because they shed new light on the relationship of Klotho with AMPAR. The Klotho-glutamate relationship appears to be bilateral, as glutamatergic neuronal activity has been shown to increase Klotho release in hippocampal neurons, and AMPAR antagonism suppresses neuronal Klotho expression (31). Importantly, AMPAR transmission may also be strongly associated with inflammation. Chronic stress-induced IL-1β production led to AMPAR internalization in hippocampal neurons (36). On the other hand, Wigerbland *et al.* showed that peripheral inflammation induced an increase in the synaptic AMPAR subunit GluA1, and intrathecal pretreatment with a TNF-blocker (Etanercept, 100 μg) reversed these effects (75). Some studies have reported that pro-inflammatory cytokines (Il-1β, TNF-α) modulate not only AMPAR subunit expression but also their phosphorylation, thereby inhibiting excitatory glutamate neurotransmission, mainly by reducing AMPAR synaptic localization (76-79). Our results equally strengthen previous reports. Notably, AMPAR levels can also be regulated by AMPK (80-81), whose reduced activity (measured as p-Thr-172-AMPK level) was demonstrated in our study and positively correlated with AMPAR protein levels. AMPK is considered a master regulator of metabolism because it maintains cellular energy homeostasis in various tissues (including the brain), and its abnormalities can result in various pathologies. Recent reports indicate that Klotho can strongly regulate AMPK expression and its activity. Klotho deficiency reduces AMPKα activity (manifested as a change in phosphorylation), which was demonstrated both in Klotho deletion mice and after the use of selective inhibitors of this protein (28, 83-85). Together, these observations indicate that multiple molecular pathways are involved in the biological function of Klotho.

In summary, this study is the first to describe alterations in a-Klotho levels in suicide victims with an accompanying inflammatory response, changes in AMPAR arrangement, and reduced AMPK activation. Our findings will not only allow us to better understand the role of a-Klotho in the molecular basis of suicide but also to set new directions in neurobiological research in the search for new targets for the development and treatment of suicide-related disorders. Assessment of the Keap1-Nrf2-IL-1 and AMPAR pathway's intracellular regulation requires further detailed research (both preclinical and clinical). Understanding the role of the Nrf2-related pathway and Klotho regulation in inflammatory responses may improve the prediction and monitoring of stress-related disorders (including suicide). The indicated signaling pathways demonstrated potential pharmacological benefits and provided a basis for new clinical trials.

At the same time, we are aware of several limitations of this study. First of all, we do not have information about possible existing mental disorders in the group of suicides. We only know that the suicide victims included in the study did not chronically use antidepressants. On the other hand, DDs are known to increase the risk of suicide, which makes it impossible to deny such a diagnosis. This hypothesis is strengthened by an increasing number of studies pointing to a similar direction of biochemical changes in FCX in patients with MDD and suicide victims (67, 86). As *Table 1* shows, three of the 14 suicides died as a result of an overdose of a combination of different substances (doxepin + clomipramine; hydroxyzine + perazine; diazepam + ethanol), but our analysis did not reveal any significant differences between the results obtained from these 3 subjects compared to the remaining ones. This allows us to assume that a single, incidental overdose of drugs does not significantly affect the cellular mechanisms we study. Therefore, based on the conducted research, we can propose a new hypothesis about the regulation of Klotho in suicidal behavior or suicide-related disorders. Other important limitations of our study are the lack of knowledge about potential comorbidities in the suicide group, the post-mortem interval (PMI) and the pH of all tissues used in this study. Finally, the small size of the groups (and disproportions in the size of both groups), as well as the gender diversity in the groups, made it impossible to analyze the changes taking into account the differences between the sexes.

Our study shows that a reduction in Klotho levels in brain structures related to mood disorders (FCx, HP) strongly correlates with suicidal behavior. As we have previously discussed extensively in our review (14), many molecular pathways are involved in the biological function of Klotho. In the context of suicide-related disorders, the most important seems to be the relationship between the Klotho protein and Keap1-Nrf2-related immune response, AMPA receptor trafficking, and AMPK activity, which is robustly confirmed by the obtained results. In addition, further studies (mainly in animal models) are necessary to elucidate the role and intracellular regulation of Klotho-Nrf2 immune responses and AMPA receptors in the molecular background of suicidality.

Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AMPK, AMP-activated protein kinase; DDs, depressive disorders; FCx, frontal cortex; GluA1, AMPAR type subunit 1; GluA2, AMPAR type subunit 2; HP, hippocampus; IL-1α, interleukin 1alpha; IL-1β, interleukin 1beta; Keap1, Kelch-like ECH-associated protein 1; NFKB-p65, nuclear factor-kappaB p65 subunit; Nrf2, nuclear factor erythroid 2-related factor 2; p-S831-GluA1, GluA1 phosphorylated at Serine 831; p-S845-GluA1, GluA1 phosphorylated at Serine 845; p-S40-Nrf2, Nrf2 phosphorylated at Serine 40; p-T172-AMPKα, AMPK phosphorylated at Threonine 172.

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